JUN 1 8 1947

VOL. XXXVI May, 1947 No. 5

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXVI | *MAY, 1947* | NUMBER 5 | CONSECUTIVE NO. 10

Profile Evaluations of Germicides*

By A. J. SALLE and B. WESLEY CATLIN

Results are presented of a comparative study to demonstrate germicide performance with respect to activity in the absence of bacteriostasis, speed of action, penetrability, tissue toxicity, amount of inactivation by organic matter, etc. It is recommended that any disinfectant being evaluated, especially one intended for clinical use, be subjected to a battery of selected tests in an effort to obtain a "profile" of its general character and efficiency under a variety of conditions.

NUMEROUS technics have been advocated in a common effort to develop adequate methods for evaluating germicides. One of the most widely used has been the U. S. Food and Drug Administration (F.D.A.) phenol coefficient method (1). Although it has been recognized that the F.D.A. method was applicable only to substances chemically related to phenol, almost every type of compound has been evaluated by this procedure.

Inhibition of growth of viable organisms in relatively high dilutions of the germicide is characteristic of mercurials and certain other preparations. To overcome this bacteriostatic action, it was recommended (1) that subcultures be made into large volumes of medium or that 4 loopfuls of the first subculture be transferred to a second tube of broth. Work by Brewer (2), Fildes (3), Heinemann (4), Nungester, Hood, and Warren (5), and others has demonstrated the desirability of incorporating a sulfhydryl-containing compound in the subculture medium used in testing mercurials. In the present study tests were run to determine the relative merit of thioglycollate over nutrient broth as a subculture medium.

The phenol coefficient test even when modified to give accurate evidence of bactericidal activity remains limited as a method of evaluation. Germicides vary not only with respect to effectiveness in killing bacteria, but also in their toxicity to host tissue, rapidity of germicidal activity, extent of inactivation by organic matter, penetrability, and activity at different temperatures.

Thus far no single method has been developed which will demonstrate the relative versatility of a disinfectant. To obtain an adequate "profile" of a germicide, a battery

* Received Jan. 20, 1947, from the Department of Bacteriology, University of California at Los Angeles.

of tests must be undertaken to determine the efficiency of the agent with respect to various desirable characteristics.

A group of commercial germicides was selected for use in a comparative study of this nature. Preliminary findings are presented.

EXPERIMENTAL

The germicides selected included the following: Solution of Iodine, U. S. P. XII (1:50); Mild Tincture of Iodine, U. S. P. XII (1:50); "Tincture of Mercresin" (1:500); "Solution of Merthiolate" (1:1000); "Tincture of Merthiolate" (1:1000); "Solution of Metaphen" (1:500); "Tincture of Metaphen" (1:200); and aqueous phenol (1:20). The concentration of "Mercresin" was considered to be 1:500, since the preparation consists of two active ingredients, secondary amyltricresols (1:1000) and ortho-hydroxyphenylmercuric chloride (1:1000). The dilutions reported in the tables refer to actual concentrations of the active ingredients.

Staphylococcus aureus of a standard phenol resistance constituted the test organism. Every effort was made to maintain the uniformity of this culture. The stock was carried on agar slants, and subcultured once a month. Following preliminary subculturing in F.D.A. broth for five consecutive days, the culture was available for use for a period of thirty days if transferred daily. In order to minimize experimental variation from day to day, it was found necessary to standardize the test suspension. To accomplish this, cultures exactly twenty-four hours old were used. Following rapid and repeated expulsion from a pipette to break up clumps of cells, the suspension was diluted with sterile broth until its optical density, as measured in a photoelectric colorimeter, was exactly equivalent to that of a No. 7 McFarland barium sulfate standard (prepared by mixing 7 cc. of 1% barium chloride with 93 cc. of 1% sulfuric acid).

In addition to the F.D.A. broth, sodium thioglycollate and sodium thiosulfate media were used for detecting viability of Staphylococcus aureus after exposure to the germicide dilutions. For subculturing from mercury-containing germicides, a 0.15% thioglycollate semi-solid broth was used, composed of Bacto Dehydrated Nutrient Broth, 4 Gm.; Bacto Dehydrated Fluid Thioglycollate Medium (Linden), 17.75 Gm.; sodium thioglycollate, 1.25 Gm. per L. Subcultures from the non-mercury-containing germicides (iodine and phenol) were made into 0.025% thioglycollate semi-solid broth similar to the previous medium except for the omission of additional sodium thioglycollate. A further type of semisolid medium, consisting of F.D.A. broth with 0.025% agar and either 1:1000 or 1:2000 sodium thiosulfate, was employed in the iodine tests. These media were tubed in 15-cc. amounts.

A technic conforming to the F.D.A. method was used to find the highest dilution of germicide capable of inhibiting growth of Staphlococcus aureus at 37°. The basic procedure was modified as required. In determining the extent of bacteriostasis, simultaneous transfers from the inoculated test dilutions were made into 10-cc. and 100-cc. quantities of F.D.A. broth and also into sodium thioglycollate or sodium thiosulfate broth. The results are shown in Table I.

TABLE I.—EFFECT OF GERMICIDES ON STAPHYLOCOCCUS AUREUS IN 10 MIN. AT 37°

Germicide	Highest Dilutions of Germicides Showing No Growth, When Subcultured into F.D.A. Broth and Thioglycollate Broth, and Their Corresponding Phenol Coefficients					
	10 Cc. F.D.A. Broth	Phenol Coefficient	100 Cc. F.D.A. Broth	Phenol Coefficient	Thioglycollate Broth	Phenol Coefficient
Iodine						
Aqueous, 1:50	1:20,000	235	1:20,000	235	1:20,000	235
Tincture, 1:50	1:20,000	235	1:20,000	235	1:20,000	235
Mercresin						
Tincture, 1:500	1:35,000	412	1:30,000	353	1:19,000	224
Merthiolate						
Aqueous, 1:1000	1:5000	59	1:1200	14	1:1000 did not kill	...
Tincture, 1:1000	1:7500	88	1:4500	53	1:4500	53
Metaphen						
Aqueous, 1:500	1:30,000	353	1:4000	47	1:500 did not kill	...
Tincture, 1:200	1:8000	94	1:4000	47	1:1200	14
Phenol						
Aqueous, 1:20	1:85	...	...	...	...	...

These findings confirm the necessity of using a neutralizing medium when testing bacteriostatic agents, such as the mercurials. This need was most apparent in the case of the aqueous solution of Metaphen. It was found to have an average growth-inhibiting dilution of 1:30,000 at 37°, as determined from transfers into 10-cc. quantities of F.D.A. broth, and 1:4000 when subcultured into 100-cc. portions of broth, but with 0.15% thioglycollate broth, the 1:500 commercial solution could

not be depended upon to kill Staphylococcus aureus under the least demanding test conditions.

Thus with the mercurials, the phenol coefficients derived from readings of absence of bacterial growth in 10-cc. broth tubes is seen not to present a valid estimate of relative germicidal value. Nor is the use of a larger volume of subculture broth adequate to neutralize traces of mercury-containing agents.

Growth-inhibiting dilutions of iodine, as determined from F.D.A. or thioglycollate broth were found to be identical with those derived from series subcultured into broth containing sodium thiosulfate in concentrations of 1:1000 and 1:2000. Since this agent effectively inactivates iodine, it is assumed that the absence of growth in subculture tubes represents true killing values, with no bacteriostasis involved.

In view of the difference in antibacterial action between the aqueous and tincture preparations of Metaphen and Merthiolate, the possibility was considered that germicidal effects of the tinctures might be largely due to the alcohol-acetone content (50% alcohol + 10% acetone). Accordingly a solution was prepared consisting of 50 cc. absolute alcohol and 10 cc. acetone made up to 100 cc. with water. This solution was tested by the F.D.A. technic at 37° and found to kill Staphylococcus aureus in ten minutes in a dilution of 1:2.5, a 20% concentration of alcohol. Whereas this alcohol-acetone mixture could be diluted only 2.5 times before losing its germicidal effect, the tinctures of Merthiolate and Metaphen, under similar conditions, retained their efficacy in dilutions of 4.5 and 6 times, respectively.

The explanation of the superior germicidal activity of the tinctures must lie in other factors, as, for example, increased stability of the commercial mixtures, or the lower surface tensions which permit greater wetting and penetration of bacterial-cell surfaces.

The apparent discrepancy between the average growth-inhibiting dilution of 1:30,000 for aqueous Metaphen and 1:8000 for the tincture subcultured in 10 cc. F.D.A. broth is believed to be due to the larger sized inoculum picked up on the planting loop in the former case. The fact that a similar discrepancy was not encountered with Merthiolate may be explained by the inclusion, in the commercial aqueous preparation, of 0.1% Monoethanolamine, a surface tension depressant.

It is interesting to note the relative efficiency of the germicides tested as judged by the number of times the commercial preparations may be diluted before losing their germicidal ability. This dilution factor was 400 for 2% aqueous and tincture of iodine, U. S. P., 38 for 1:500 tincture of Mercresin, 4.5 for 1:1000 tincture of Merthiolate, and 6 for 1:200 tincture of Metaphen.

Tests were run to determine killing dilutions of the germicides on Staphylococcus aureus at 20°. The F.D.A. method was employed with the exception that subcultures were made into thioglycollate broth. Results are shown in Table II.

TABLE II.—EFFECT OF GERMICIDES ON STAPHYLOCOCCUS AUREUS IN 10 MIN. AT 20°

Germicide	Highest Dilutions of Germicides Showing No Growth, When Subcultured into Thioglycollate Broth, and Their Corresponding Phenol Coefficients: Thioglycollate Broth	Phenol Coefficient
Iodine		
Aqueous, 1:50	1:20,000	308
Tincture, 1:50	1:20,000	308
Mercresin		
Tincture, 1:500	1:13,000	200
Merthiolate		
Aqueous, 1:1000	1:1000 did not kill	...
Tincture, 1:1000	1:2000	31
Metaphen		
Aqueous, 1:500	1:500 did not kill	...
Tincture, 1:200	1:1100	17
Phenol		
Aqueous, 1:20	1:65	...

In tests to determine the influence of organic matter, dilutions of the disinfectants were prepared in 4-cc. quantities and 1 cc. of 50% sterile horse serum (without chemical preservative) was added to each tube, giving a total volume of 5 cc. with a final concentration of 10% serum. The results are recorded in Table III.

It may be seen that all of the germicides were reduced in activity in the presence of organic matter, although considerable variation was noted in this respect. Mercresin and iodine were subject to greater inactivation than Merthiolate and Metaphen under test conditions.

TABLE III.—EFFECT OF ORGANIC MATTER ON GERMICIDES TESTED AGAINST STAPHYLOCOCCUS AUREUS IN 10 MIN. AT 37°

Germicide	Highest Dilutions of Germicides Showing No Growth in the Presence of 10% Horse Serum, When Subcultured into Thioglycollate Broth, and Their Corresponding Phenol Coefficients: Thioglycollate Broth	Phenol Coefficient
Iodine		
Aqueous, 1:50	1:2500	29
Tincture, 1:50	1:2500	29
Mercresin		
Tincture, 1:500	1:3750	44
Merthiolate		
Aqueous, 1:1000	1:1250 did not kill	..
Tincture, 1:1000	1:3600	42
Metaphen		
Aqueous, 1:500	1:625 did not kill	..
Tincture, 1:200	1:1100	13
Phenol		
Aqueous, 1:20	1:85	..

Tests of the variation of killing dilution with time were conducted according to the F.D.A. method except that subcultures were made at minute intervals

during a ten minute period and thioglycollate broth was used as the transfer medium. In view of the fact that the ten-minute period of exposure of Staphylococcus aureus to the germicide dilutions is somewhat arbitrary and the probability that it may considerably exceed the time during which the germicide is available in effective concentration under clinical conditions, the advisability of testing the speed of action is indicated. The results are shown in Fig. 1. The dilutions marked with a solid circle represent the killing concentrations for that particular interval of time.

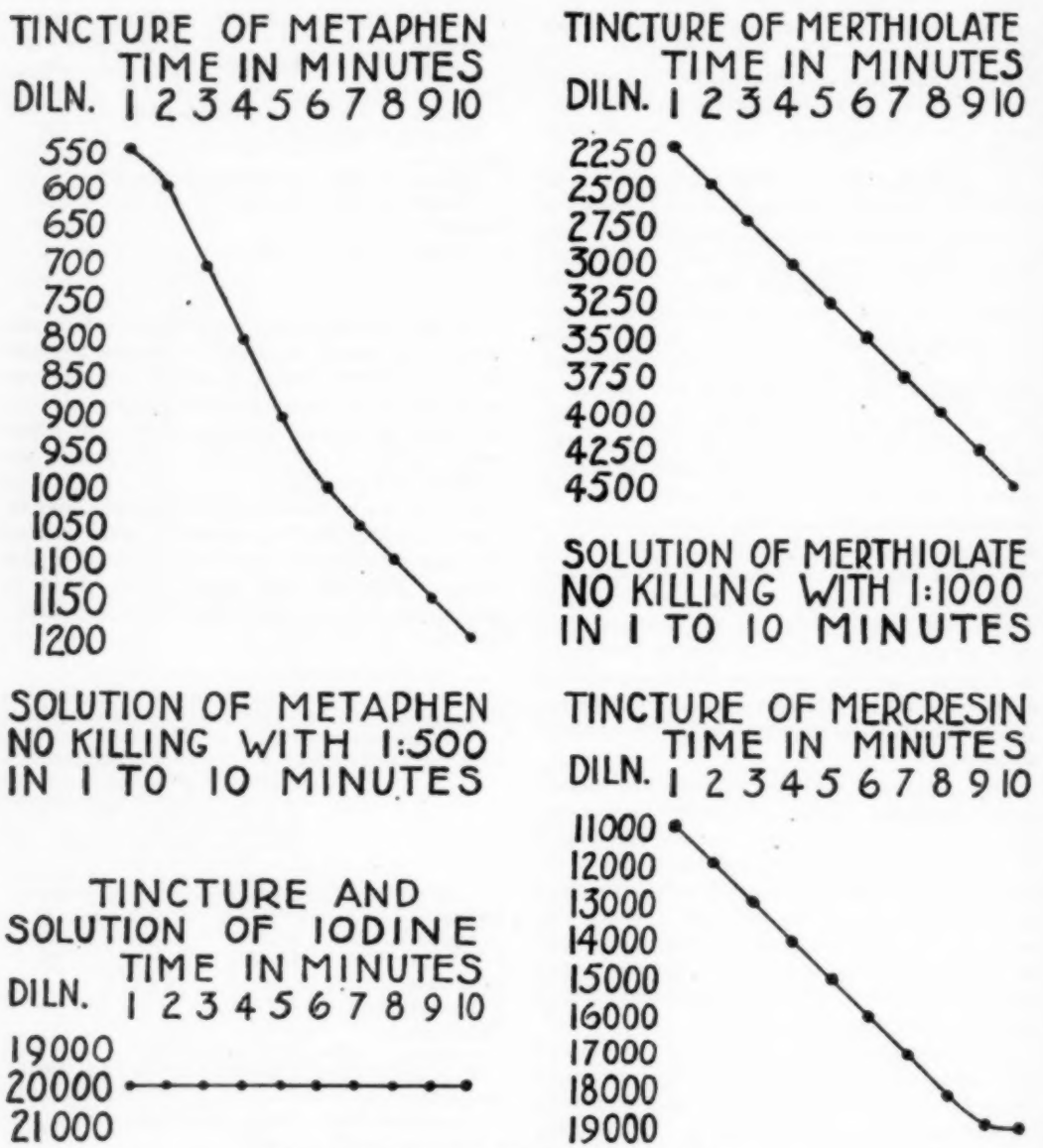

Fig. 1.—Variation of Killing Dilution with Time.

It may be seen that all of the mercurial germicides tested increased in effectiveness with time, the average killing dilution in one minute being roughly double the concentration required to kill in ten minutes. In the case of iodine, the killing dilution at one minute was the same as for ten minutes.

The agar cup-plate method is that described in the F.D.A. circular 198 (1). Plates were poured with 20 cc. quantities of 1.5% agar following inoculation with 0.1 cc. of a twenty-four hour adjusted culture of Staphylococcus aureus. After the agar had solidified, a disk was removed from the center of each plate with the aid of a sterile cork borer 1.5 cm. in diameter. Ten per cent serum plates were prepared by adding 2 cc. of sterile horse serum to 18 cc. of the agar. Stock dilutions of the test germicides were

introduced into the agar cups in 0.1-cc. quantities. Plates were incubated for twenty-four hours at 37° under unglazed porcelain Petri dish covers. Measurements were made of the zone of complete inhibition of bacterial growth. Since several of the bactericides produced zones having very hazy peripheries, it was found helpful to check measurements with a ten-diameter magnification. The results are recorded in Table IV.

TABLE IV.—RESULTS OF TESTS USING FDA AGAR CUP-PLATE METHOD

Germicide	Diameter in Cm. of Zone of Complete Inhibition of Bacteria Growth (*Staph. aureus*) F.D.A. Agar, without Serum	10% Serum Agar
Iodine		
Tincture, 1:50	4.9	3.4
Aqueous, 1:50	4.5	2.7
Merthiolate		
Tincture, 1:1000	4.1	2.1
Aqueous, 1:1000	2.5	1.8
Metaphen		
Tincture, 1:200	3.3	1.9
Aqueous, 1:500	2.9	1.9
Mercresin		
Tincture, 1:500	2.7	1.7
Phenol		
Aqueous, 1:20	2.1	2.1

The method of Salle, *et al.* (6), was used to determine the toxicity of each of the 4 tincture preparations to living embryonic chick heart fragments cultivated in vitro. Hearts were removed from thirteen-day chick embryos and minced aseptically into particles 0.5 to 1 mm. in diameter. This material was washed in saline and resuspended in Tyrode's solution in the proportion of one heart per cc.

A series of aqueous dilutions of the germicide was prepared, each tube containing a total volume of 5 cc. Following preliminary warming of all tubes in the 37° water bath, 0.5 cc. of the heart fragment suspension was added to the first dilution. The tube was shaken and returned to the bath. This procedure was continued at minute intervals for the remaining dilutions. Exactly ten minutes after addition of the tissue suspension to the first tube, the germicide was aspirated from the sedimented fragments and about 20 cc. of physiological saline was added to wash the tissue. At minute intervals subsequent tubes were similarly treated. When the fragments had settled out, the supernatant fluid was siphoned off. The tissue particles were then imbedded in rabbit plasma in Carrel flasks. The imbedding medium consists of 0.4 cc. plasma, 0.7 cc. Tyrode's solution, and 0.3 cc. embryonic extract. This latter is the clear supernatant fluid from ground twelve-day chick embryos diluted to 5 times the volume with Tyrode's solution prior to centrifugation. Following coagulation of the plasma medium, 1 cc. of embryonic extract was added as nutrient. Flasks were plugged with paraffined corks and incubated for seventy-two hours at 37°.

The highest dilution acting during the ten-minute exposure period to inhibit the subsequent proliferation of fibroblasts from over 50% of the 0.5–1.0 mm. sized heart fragments was considered to be the killing dilution. The toxicity index was calculated. This is defined as the ratio of the killing dilution of the germicide tested against embryonic tissue to the highest dilution required to kill the bacterial culture under similar test conditions. The results are presented in Table V.

TABLE V.—EFFECT OF GERMICIDES ON EMBRYONIC CHICK HEART TISSUE AND STAPHYLOCOCCUS AUREUS AT 37°

Germicide	Highest Killing Dilution for Tissue and Bacteria and Corresponding Toxicity Index: Tissue (A)	Bacteria (B)	Toxicity Index (A/B)
Iodine			
Tincture	1:4000	1:20,000	0.2
Mercresin			
Tincture	1:9000	1:19,000	0.5
Merthiolate			
Tincture	1:15,000	1:4500	3.3
Metaphen			
Tincture	1:12,000	1:1200	10.00

Theoretically, a toxicity index lower than 1 indicates relatively greater toxicity to bacterial cells than to host tissue. This differential ability of a disinfectant to exert lethal effect on bacteria in a concentration which is innocuous to the host is one of the primary considerations in the selection of a germicide for clinical use. Other important qualifications include rapidity of disinfecting action-penetrability, and germicidal efficiency in the presence of organic matter.

It is recommended that any germicide being evaluated, especially one intended for clinical use, be subjected to a battery of selected tests in an effort to obtain a "profile" of its general character and efficiency under a variety of conditions.

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Advisability of Some Proposed Changes for the Evaluation of Disinfectants*

By OTTO RAHN

The standard methods for the evaluation of disinfectants are not entirely satisfactory. Several changes have been proposed, especially since the advent of quaternary ammonium compounds. Their effect upon the results by standard methods is discussed.

THE RELIABILITY of our standard methods of evaluating germicides has been frequently challenged, and several suggestions for improvement have been made. Some investigators believe that considerable error arises from unequal numbers of bacteria under test (1, 2). Others consider the sampling by loop too inaccurate and propose to use 1 ml. or the entire suspension of 5.5 ml. in the test for survivors. Then there is the danger of bacteriostasis by transfer of too much disinfectant to the test broth, and the general worry about too much variation in the composition of the test broth.

METHODS AND DISCUSSION

The Size of Inoculum.—The effect of a change in the number of bacteria subjected to the test can be predicted by a simple calculation. Bacteria suspended in a disinfectant solution do not die all at once. As early as 1908, Chick (3) had shown that bacteria die at a constant death rate, i.e., of all bacteria still alive at the beginning of any minute, the same percentage will die during that minute. This is now called the logarithmic order of death. It is represented by the equation:

$$Kt = \log \frac{\text{inoculum}}{\text{survivors}}$$

where t is the exposure time, and K the death rate constant which is characterized by the kind of disinfectant and its concentration.

Table I shows two simple examples, namely, bacteria dying at the rate of 90% of the survivors per minute and 50% per minute, 10% and 50%, respectively, remaining alive at the end of each minute. Experimental data proving this law have been compiled by Rahn (4, p. 13).

In measuring the phenol coefficient, 0.5 ml. of a fullgrown culture is mixed with 5 ml. of the disinfectant. Since the culture contains about a billion cells per ml., the test solution has about 100 million living cells per ml. at the start.[1] The suspension is considered sterile if one loopful transferred to broth produces no growth. However, that proves only that there was less than one viable cell per loop which contains 0.01 ml. With 10 bacteria per ml. surviving, only one out of every 10 loop transfers would produce growth. Therefore, "no growth" indicates merely less than 100 viable cells per ml., and it can be averaged as representing 10 viable cells per ml.

If the above equation is applied to the phenol coefficient technique, the inoculum is approximately 100 million, the survivors approximately 10 per ml. Therefore

$$Kt = \log \frac{100{,}000{,}000}{10} = 7$$

The death time t is between five and ten minutes, and may be averaged seven and one-half minutes. The death rate constant for the phenol coefficient is then

$$K = \frac{7}{7.5} = 0.93$$

If the inoculum were twice as large, everything else being the same,

$$Kt_2 = 0.93t_2 = \log \frac{200{,}000{,}000}{10} = 7.301$$

$$t_2 = 7.8 \text{ minutes}$$

If the inoculum were only half as large,

$$Kt_m = \log \frac{50{,}000{,}000}{10} = 6.7$$

$$t_m = 7.2 \text{ minutes}$$

Thus, with the number of test bacteria varying from 1:2:4, the death times vary from 7.2 to 7.5 to 7.8 minutes. The variation in the number of bacteria in standard procedure does not exceed these limits. Pressman and Rhodes (2) found the greatest fluctuation of bacterial population in 6 parallel experiments to be 16%. It seems safe to say that a standardization of the inoculum by means of a nephelometer (which does not differentiate between living and dead bacteria) will not improve the accuracy of the experiment.

The technique of Hucker, *et al.* (5) who add 1 ml. of disinfectant to 9 ml. of culture increases the death time noticeably.

* Received Sept. 14, 1946, from the Laboratory of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca, N. Y.

[1] Pressman and Rhodes (2) report 10 billion cells of *E. typhosa* per ml. (their Table II). Such high numbers can be ordinarily obtained only by continuous aeration. The number of 560×10^{12} given in their Table I must be a typographical error.

$$t = \frac{1}{0.93} \log \frac{900{,}000{,}000}{10} = \frac{7.95}{0.93} = 8.55 \text{ minutes.}$$

Still, the increase of the inoculum by 900% increases the death time only 12%.

The Size of the Test Sample.—Whereas some investigators, fearing bacteriostasis by disinfectant carried over with the sample, use for the viability test a sample much smaller than one loop, others recommend much larger samples, even the entire contents of the medication tube.

For mercurials, Shippen (6) transferred one loop from the first subculture to a second broth tube, and used 4 loopfuls of the second tube for testing. This means a dilution of 1:25,000. With 2500 bacteria alive per ml., the second tube will remain sterile 9 times out of 10. Applying the above formula, with the same standards, the death time t can be computed.

$$0.93t = \log \frac{100{,}000{,}000}{2500} = \log 40{,}000 = 4.602$$
$$t = 4.9 \text{ minutes}$$

instead of the 7.5 minutes needed to produce sterility in the *first* transfer.

If the critical death time of any disinfectant solution tested by the F.D.A. method is 7.5 minutes, it will be, under identical circumstances, 4.9 minutes by the Shippen method, and 9.4 minutes by the Klarmann method. The first method gives a too favorable result while the second method is too severe in comparison with the F.D.A. technique.

Bacteriostasis by Carry-Over of Disinfectant.—As has been shown above, with the Shippen method a carrying over of enough disinfectant to produce bacteriostasis is feared by some bacteriologists. The danger seems rather remote if we realize that by the transfer of one loop (0.01 ml.) from the medication tube to 10 ml. of broth, the disinfectant is diluted 1:1000. No disinfectant is known where bacteriostasis is produced by 1/1000 of the bactericidal concentration. The ratio between these two concentrations has been studied by the author (4, pp. 131–33). It varies greatly, from 1:3 with $HgCl_2$ to 1:630 with phenyl mercuric nitrate, but none was found to be as high as 1:1000. However, with disinfection of spores, the concentrations needed are so high that the dilution 1:1000 is still likely to produce bacteriostasis. In that case, neutralizers or antidotes must be used.

TABLE I.—DECREASE OF VIABLE BACTERIA IN DISINFECTION

% dying per minute	50	90
% surviving per minute	50	10
Death rate constant	$K = 0.301$	$K = 1.000$
Initial number of bacteria per ml.	100,000,000	100,000,000
Bacteria killed during first minute	50,000,000	90,000,000
Bacteria alive at end of first minute	50,000,000	10,000,000
Bacteria killed during second minute	25,000,000	9,000,000
Bacteria alive after 2 minutes	25,000,000	1,000,000
Bacteria alive after 3 minutes	12,500,000	100,000
Bacteria alive after 4 minutes	6,250,000	10,000
Bacteria alive after 5 minutes	3,125,000	1,000
Bacteria alive after 6 minutes	1,562,500	100 or 1 per loop
Bacteria alive after 7 minutes	781,250	10 or 1 per 10 loops
Bacteria alive after 8 minutes	390,625	1
Bacteria alive after 9 minutes	195,312	0.1 or 1 per 10 ml.
Bacteria alive after 10 minutes	97,656	1 per 100 ml.

Heinemann (7) found that the Shippen retransfer technique gave falsely high killing dilutions. He explains this by ineffective elimination of bacteriostasis, but the result may be largely due to the fact that this method removed living bacteria not only by killing, but also by dilution.

The opposite recommendation has been made in case of quaternary ammonium compounds. In order to avoid large volumes, Klarmann and Wright (8) use a total medication volume of only 0.55 ml. and mix this with 20 ml. of broth. "No Growth" in this case means less than 2 survivors per ml. Assuming 0.2 survivors per ml. being a safe average, our equation is

$$0.93t = \log \frac{100{,}000{,}000}{0.2} = \log 500{,}000{,}000 = 8.7$$
$$t = 9.4 \text{ minutes}$$

instead of the 7.5 minutes required if the standard loop transfer method were used.

Application of Antidotes, Antagonists, Neutralizers, etc.—Sterilization of bacteria is brought about by a chemical reaction of the disinfectant with some vital part of the cell. This reaction requires time. It may take place in several steps of which the first step may be reversible. After five minutes' contact with the disinfectant solution, all cells contain a certain amount of disinfectant, but this may not have produced as yet the fatal reaction. Transfer to fresh broth may not induce the disinfectant to diffuse quickly out of the cell, and the cell may die in the new broth although it was not dead at the moment of transfer. In such a case, the immediate application of an antidote would save the life of bacteria which would be considered dead by the standard technic.

For example, Gegenbauer (9) found that staphylococci treated with $HgCl_2$ can be prevented from remaining sterile by repeated washing, provided that the contact with the poison has been less than an

hour. After that time, no amount of washing can save the cells, for the mercury ions have reacted with some cell constituents. But H_2S may still reverse this chemical reaction by forming insoluble HgS within the cell (Fig. 3). This reversion which restores the power of reproduction is still possible after sixteen, but not thirty-three hours if the original disinfectant contained 1% $HgCl_2$. If only 0.05% had been used, reversion was possible after seventy-two, but not after 101 hours. Valko and DuBois (10) treated bacteria "sterilized" by cationic detergents with anionic detergents, and if this was done within thirty minutes after exposure, the bacteria became capable of reproduction.

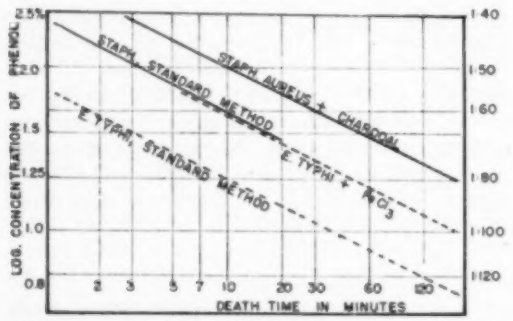

Fig. 1.—The effect of charcoal on the death time of staphylococci, and of ferric chloride on the death time of typhoid bacteria with phenol.

Many similar observations have been published, and they demonstrate that the borderline between bacteriostatic and bactericidal concentrations is not at all definite. It depends upon the treatment of the bacteria after exposure. Bacteria, which after disinfection are unable to grow in broth, may still be able to multiply in the intestine, or in wounds. Yeasts apparently killed by an antiseptic may grow again in a medium of more favorable *p*H.

The velocity of reaction between poison and antidote is important. In the case of mercury salts and hydrogen sulfide, the reaction between the ions is instantaneous whereas formaldehyde reacts so slowly with ammonia that after-treatment of formalin-killed bacteria with ammonia does not revive them.

Cationic detergents are rapidly adsorbed on the cell surface (11), resulting in a high diffusion potential which accelerates the fatal reaction. When a cell is transferred from Benzalkonium Chloride (Zephiran Chloride) solution into broth, the adsorbed disinfectant will but slowly leave the cell, and the probability is great that the detergent continues to enter the cell for a while until equilibrium in the new broth is established. If a chemical antidote Sodium Lauryl Sulfate (Duponol) is added which changes all the detergent on the surface quickly to a harmless compound, further fatal reaction is prevented, and the cells retain whatever reproductive power is left, though they might have lost it without the antidote.

Charcoal removes adsorbed compounds physically, and therefore acts more slowly than a chemical antidote. A reversion of chemical reactions with the cell contents by charcoal is not possible. Nevertheless, the cell surface is cleared from excess detergents, and bacteria are saved which without charcoal would have become sterile.

Even phenol is subject to "detoxication" by charcoal, according to the report by Flett, *et al.* (12). While the standard method required dilution 1:60 to kill, charcoal changed it to 1:50. Translated into death times, this means that charcoal increased the death time of 1:60 phenol from ten minutes to about twenty-five minutes.

The three graphs represent the change of deathtime with the concentration of the disinfectant. On a double logarithmic scale, the relationship is rectilinear (4, p. 70). The first graph represents the data by Flett, *et al.* with phenol where the killing concentration had to be increased when the bacteria were treated with charcoal or ferric chloride after exposure. It represents not a true revival, but the prevention of the lethal reaction by prompt and complete removal of the disinfectant. The second graph shows the author's results with Zephiran acting on *Staphylococcus aureus*. With 60 p. p. m. of Zephiran, the bacteria died between seven and fifteen minutes, when transferred to broth after treatment. When the broth contained charcoal, the deathtime increased to nineteen minutes. When Duponol was used as antidote, the cells did not succumb until after twenty-three minutes. Again, this is prevention of the lethal reaction, not revival.

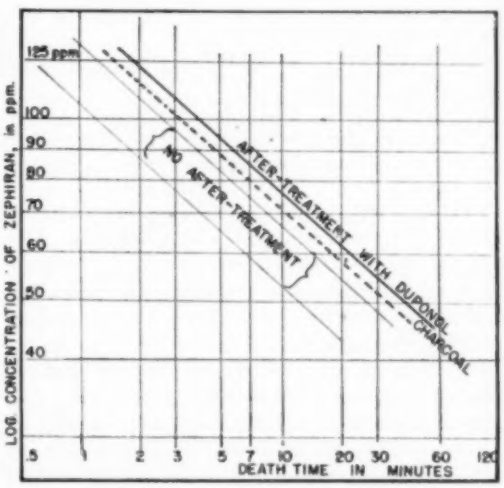

Fig. 2.—The effect of charcoal and Duponol on the death time of staphylococci with Zephiran.

The third graph shows Gegenbauer's data on the effect of mercuric chloride with after-treatment by hydrogen sulfide on staphylococci. Gegenbauer proved conclusively that the reaction of $HgCl_2$ with some cell constituent was reversed by H_2S.

This is a true example of reversion, an actual revival, not merely prevention of the lethal reaction. Therefore the time scale of this graph is hours while the others have a scale of minutes.

It has been suggested that the standard method for the evaluation and comparison of disinfectants should require the use of antidotes. This is not feasible because different disinfectants would require different antidotes, and their relative efficiency could not be the same. New antidotes will be found against old disinfectants, and their evaluation will be changed. The observation by Flett, *et al.* mentioned above has shaken the reliability of phenol as a standard. It seems impossible to develop a standard method which applies antidotes. It is important, however, to know under what conditions a disinfectant loses its power. The statement on containers of cationic detergents that they are incompatible with soap is very commendable.

Detoxication during Exposure.—No bacteriologist would determine the phenol coefficient of $AgNO_3$ by using a culture grown in the standard F.D.A. broth because it contains 0.5% NaCl which would precipitate and inactivate all silver ions instantaneously. However, with complex organic compounds, similar situations may arise unintentionally. [The 0.5 ml. of broth culture added to the disinfectant amounts to 1500 p. p. m. of organic matter (4, p. 115) which greatly decreases the efficiency of such disinfectants as chlorine, iodine, permanganate, or mercury salts. When washed cells are tested, these disinfectants are much more efficient.] Traces of *p*-amino benzoic acid in broth will interfere with sulfonamides (13). The small amounts of lecithin and other lipids in broth inactivate quaternary ammonium compounds (14). The proportion of lipids and nutrilites is not the same in different peptones, not even in different batches from the same manufacturer, and this is one of the causes of diverging results (15).

Cationic detergents are precipitated by anionic detergents. When equivalent solutions of Zephiran and Duponol were mixed, the surface tension was very nearly that of water. However, the reaction

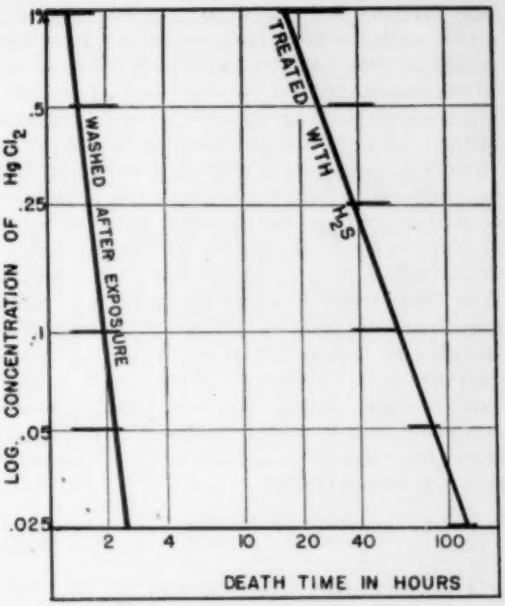

Fig. 3.—The effect of hydrogen sulfide on the death time of staphylococci with mercuric chloride.

product was bacteriostatic, as the following experiment shows. *Staph. aureus* grows normally in broth with 10 p. p. m. Duponol. This amount is equivalent to 12 p. p. m. Zephiran, but no growth could be obtained when the two were mixed. When smaller amounts of Zephiran were added to broth + 10 p. p. m. Duponol, the results shown in Table II were obtained, the numbers indicating on what day growth was first observed.

Zephiran can be removed from broth by adsorbent charcoal (Norit A). The adsorption was measured by sterilizing, in 10-ml. portions, broth containing 1% Norit, and adding measured amounts of concentrated Zephiran solution, and eventually also of Duponol solution (Table III). The adsorption was not instantaneous. As a rule, no growth was obtained when the Norit broth was inoculated half an

TABLE II

Time Allowed before Inoculation, Hr.		Parts per Million										
	Zephiran:	0	2	3	4	5	6	7	8	9	10	12
	Duponol:	10	10	10	10	10	10	10	10	10	10	10
		Days										
2	Growth after	1	1	..	4	..	7	..	∞	..	∞	∞
4	Growth after	1	1	1	1	2	3	5	7	9	∞	∞

TABLE III.—GROWTH OF STAPHYLOCOCCI WITH ZEPHIRAN IN BROTH AND IN MILK WITH ADDITIONS OF LECITHIN, CHARCOAL, AND DUPONOL

Zephiran, p. p. m.	20	50	80	100	120	160	200	300	500	1000
Broth + 1% lecithin	+	+		0			0		0	
Broth + 1% Norit charcoal			+	+	+	+	+	0	0	0
Same + equivalent Duponol			+	+	+	+	+	+	0	0
Skim milk			+		0	0	0	0	0	0
Same + equivalent Duponol			+	+	0	0	0	0	0	0

hour after 80 to 100 p. p. m. Zephiran had been added, while five hours later, even 200 p. p. m. permitted growth. As 40 p. p. m. kill staphylococci within ten minutes and 4 p. p. m. are bacteriostatic, this result indicates that complete removal of large amounts of Zephiran requires considerable time. Growth in concentrations of 200 p. p. m. or more was delayed for three days, indicating that small amounts (less than 4 p. p. m.) had not been adsorbed. Duponol in combination with Norit removed more Zephiran than Norit alone, but there was a definite limit. Milk removed at least 75 p. p. m. of Zephiran from solution, either by adsorption on the casein particles, or perhaps partly by chemical reaction with milk lipids. Duponol had little additional effect. A small amount of lecithin added to broth removed at least 45 p. p. m. Zephiran. The antagonistic effect of soap or sodium stearate is probably similar to that of lecithin.

The amount of Zephiran removed by bentonite, filter paper, and yeast cells was found to be considerable (11). Probably even the walls of the glassware adsorb some of these compounds. All surface tension depressants are concentrated in foam. These and perhaps other properties of detergents may be in part the cause of the difficulties encountered in the assay of their germicidal value. They are of greatest importance for the application of quaternary ammonium compounds in medicine or industry.

The presence in the culture medium of compounds which may counteract certain types of disinfectants is unavoidable in some cases. None of the culture media proposed as substitutes for F.D.A. broth will remain neutral to *all* disinfectants. The alternative is the use of washed cells (4, p. 110). The effect of various types of organic matter can be determined separately to fit the intended application.

SUMMARY

The fluctuations in the number of cells subjected to disinfection tests are so small, as long as standard methods are followed, that they cannot influence the result. A doubling of the cell number would change the death time from 7.5 to 7.8 minutes; a tenfold increase would prolong it to 8.6 minutes. Such great variations in cell numbers do not occur under standard conditions.

Some of the changes recommended in the size of the sample for ascertaining viability affect the result decidedly. By the Shippen technique, viable bacteria disappear after 4.6 minutes when by the standard method, the death time is 7.5 minutes. The Klarmann-Wright method gives a death time of 9.4 minutes instead of 7.5 by standard technique.

The application of antidotes cannot be incorporated in a standard method because efficient, quickly reacting antidotes are not known for all disinfectants. The discovery of better antidotes would upset all standards.

Two modes of action must be distinguished with antidotes. They may merely remove the excess disinfectant from the broth and cell surface as, e.g., charcoal does with cationic detergents or phenol, or they may diffuse into the cell and reverse the reaction between disinfectant and cell content as e.g., H_2S does with $HgCl_2$. In this latter case, the cells are truly revived, and this revival reaction may be possible a day or two after the poisoning. In the former case, the antidote merely prevents the fatal reaction from taking place, but does not reverse the reaction where it already has occurred. These antidotes are ineffective unless applied very soon after poisoning.

The media used in standard methods do not remove or neutralize disinfectants intentionally, but the organic matter of the broth reacts with heavy metals, chlorine, iodine, and cationic detergents, and thus affects their phenol coefficient greatly. It is doubtful that a synthetic medium can be found that would not react with some of the disinfectants.

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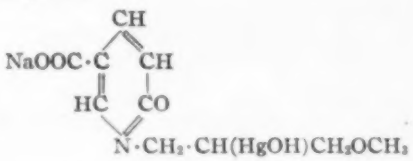
On the Potency and Toxicity of a New Mercurial Diuretic: Sodium N-(beta-Hydroxymercuri-gamma-Methoxypropyl)-2-Pyridone-5-Carboxylate*

By RAYMOND L. CAHEN†

The diuretic activity and toxicity of a new organic mercurial compound, Sodium N-(β-mercuri-γ-methoxypropyl)-2-pyridone-5-carboxylate, have been studied in different animal species. Comparisons of this compound in combination with theophylline with mersalyl-theophylline were made. The results of these studies using cats, rats, and rabbits and different routes of administration are reported.

A NEW organic mercurial described in 1942 by Hunt, Walter, and Fosbinder (1), and consisting of a pyridone nucleus joined with a mercurated side chain through an amide linkage has been investigated. This compound is Sodium N-(β-hydroxymercuri-γ-methoxypropyl)-2-pyridone-5-carboxylate.



Inasmuch as this compound is structurally related to an existing mercurial diuretic, and because a preliminary study proved that it is rapidly absorbed from the site of injection with little retention of mercury by the body (1), the assay of its diuretic activity has been made in different animal species. The study of this compound in combination with theophylline[1] which is reported to enhance the absorption and decrease the local reaction of other mercurials (3, 6), is herein described. The safety margin of this compound was determined by a study of its acute toxicity and compared with Mersalyl-Theophylline injection.

* Received Oct. 10, 1946, from the Department of Pharmacology, The Maltbie Chemical Company, Newark, N. J.
† Grateful acknowledgment is made to Dr. Carl Schmidt, Dr. Chauncey Leake, and Dr. Michael Mulinos for critical advice. The author is also indebted to Dr. R. J. Fosbinder, Dr. W. Modell, Dr. S. Krop, and Dr. N. Ercoli for many opportunities of discussion.

[1] This combination will be designated as HMP.

EXPERIMENTAL

Material.—Sodium N-(β-hydroxymercuri-γ-methoxypropyl)-2-pyridone-5-carboxylate (HMP) has been used in combination with theophylline in the ratio of 1:0.42 by weight.[2] A 10% aqueous solution was prepared, which is a colorless, bitter liquid with a slightly alkaline reaction. One cubic centimeter of the solution contains 0.10 Gm., representing 0.0469 Gm. of mercury and 0.042 Gm. of theophylline. Mersalyl and Theophylline injection (commercial Salyrgan-Theophylline solution) has been used representing a combination of Mersalyl with Theophylline in the ratio of 1:0.50 by weight. One cubic centimeter of solution contains 0.10 Gm. Mersalyl representing 0.0396 Gm. of mercury and 0.05 Gm. of Theophylline.

I. Diuretic Activity

A. Method.—The diuretic activity was determined in 3 animal species.

(*a*) *Rat.*—Using the technique of Lipschitz (2) 5 groups of 16 animals were employed for the determination of diuretic potency after selection of the optimal dose. Food and water were withheld overnight. The next day all rats received orally 25 cc./Kg. of saline. Immediately thereafter, 2 groups were injected intramuscularly with 20 mg./Kg. of HMP; 2 groups received intramuscularly the standard (20 mg./Kg. of Mersalyl-Theophylline injection), while the fifth, serving as a control, received saline alone. At least one week was allowed before the rats were used again. During the following weeks, the groups were crossed over so that those, which had received HMP, received either Mersalyl and Theophylline injection or saline.

(*b*) *Cat and Rabbit.*—The same method has been used in 30 cats and 32 rabbits except that before administration of saline or drug the bladder was emptied and the urine discarded. Twenty-four hours after administration of the drug, the bladder was again emptied and the urine added to the quantity collected from the metabolism cage. The method is similar to that described by de Bodo, *et al.*, for the study of antidiuretics (9).

B. Results.—(*a*) *Rat.*—Preliminary information, obtained by using 10, 20, 30, and 40 mg./Kg. of compound, showed that the dose which was effective, without inducing either toxic effects or a decrease in diuretic action, was 20 mg./Kg. for both

[2] The compound was prepared by Dr. L. A. Walter of our Laboratories.

drugs. The optimal period for urine collection appeared to be twenty-four hours instead of five hours, as indicated by an increase in the rate of excretion after five hours. The results of the determination of diuretic potency of 20 mg./Kg. of drug, i.e., 7.92 mg./Kg. of mercury as Mersalyl-Theophylline and 9.32 mg./Kg. mercury as HMP, are summarized in Table I. Data reported concern the mean diuretic response obtained with each drug compared with the saline control. The diuretic activity is given by the ratio: urinary excretion in test group/urinary excretion in control group. Table I shows that HMP had approximately the same diuretic activity (1.25) as Mersalyl-Theophylline (1.31) administered by the same route and at the same dose level of 20 mg./Kg.

(*b*) *Cat.*—Preliminary experiments indicated that the optimal diuretic dose of HMP and Mersalyl-Theophylline was 15 mg./Kg. intramuscularly; 5.94 mg. of mercury for Mersalyl-Theophylline, and 7.04 mg. mercury for HMP. Table I summarizes the results of the determination of comparative diuretic potency of these drugs at the above dose level. As can be seen, the diuretic activity of HMP (3.65) as determined by these experiments was 30% less than with Mersalyl-Theophylline (5.27).

II. Toxicity

Inasmuch as the usefulness of any diuretic is strictly dependent upon its toxicity, it was essential to determine the acute toxicity of HMP by intravenous and intramuscular routes of injection. Because of the possible variation in toxicity and metabolism between different species, a study of the acute toxicity was made in 3 species of animals, namely, the rabbit (1, 6), the cat, commonly used for testing various mercurial diuretics (4, 5, 7), and the rat. In this study Mersalyl-Theophylline was selected as a standard of comparison because it had been previously investigated in detail and has enjoyed widespread clinical use.

(1) Intravenous Toxicity.—Two types of methods were used: (1) a rapid single injection and (2) slow continuous perfusion until death. Because aging was found to decrease the toxicity of Mersalyl-Theophylline injection (4), the toxicity of a three-month-old HMP solution was determined in the cat.

A. Methods.—A determination of the percentage mortality after single intramarginal injection in the rabbit has been made using 2 different concentrations and injection rates; a 10% solution injected in a period of one minute (1), and a 1% solution injected

TABLE I.—DIURETIC ACTIVITY OF MERCURIALS FOLLOWING INTRAMUSCULAR ADMINISTRATION

	Urinary Excretion: Volume Urine × 100 / Volume Liquid Administered						Diuretic Activity: Urinary Excretion of Test / Urinary Excretion of Control			
	Mersalyl-Theophylline		HMP		Controls		Mersalyl-Theophylline		HMP	
I Rat (20 Mg./Kg. Drug)										
Minimum	90	75.5	82.5	98	73	85	1.20	0.88	1.25	1.07
Maximum	141	142	132	125	100	97	1.57	1.44	1.58	1.40
Average (80 rats)	118	110	123	96.7	86.8	88.2	1.36	1.30	1.40	1.07
Mean ± S.E.	119 ± 6.3		110.3 ± 4.5		87.7 ± 5.5		1.31 ± 0.06		1.25 ± 0.06	
P[a]	0.30						0.05			
II Cat (15 Mg./Kg. Drug)										
Minimum	117	145	65.7	37	6.4	25	1.17	2.2	1	0.57
Maximum	321	440	244	300	43.2	72	10	8.8	8.9	4.6
Average (15 cats)	209	268	151	162	19.7	54.1	5.35	5.20	4.2	3.08
Mean ± S.E.	238 ± 5.7		156 ± 3.3		37.3 ± 6.3		5.27 ± 0.57		3.65 ± 0.65	
P[a]	0.10						0.10			
III Rabbit (5 Mg./Kg. Drug)										
Minimum	46	110	30	120	86	31	0.32	1.23	1.07	1.35
Maximum	194	320	230	300	280	250	2.06	10	3.4	9.6
Average (16 rabbits)	177	258	153	194	120	123	1.13	5	2.18	4.20
Mean ± S.E.	187 ± 30.4		173 ± 39.9		121 ± 55.0		3.25 ± 1.0		3.20 ± 0.9	
P[a]	0.80						0.70			

[a] Probable coincidence calculated according to the method described by R. A. Fisher (12).

(*c*) *Rabbit.*—A dose of 10 mg./Kg. of each compound intramuscularly produced a definite decrease in urinary excretion as compared to the controls while a dose of 15 mg./Kg. appeared to be toxic. At a dose level of 5 mg./Kg. (1.98 mg./Kg. mercury as Mersalyl-Theophylline and 2.34 mg./Kg. mercury as HMP), however, the results given in Table I show that HMP had the same diuretic activity as Mersalyl-Theophylline.

over a period of three minutes (5), the rate of injection being decreased in the latter case in order to avoid sudden death by speed shock (10). The toxicity was evaluated by injecting a 10% solution in the saphenous vein over a period of three minutes. In the rat, 0.5%, 1.0%, and 2.0% solutions were injected in the tail vein. In every instance all deaths observed within ten days were recorded.

Slow intravenous infusion was performed in the

marginal vein of the rabbit and the femoral vein of the cat at a concentration of 0.5%. Urethane (1 Gm./Kg. intraperitoneally) was used as the anesthetic in the cat; all other experiments were done on unanesthetized animals.

The median lethal dose (LD_{50}) has been evaluated according to Behrens (14). In addition, the method of Miller and Tainter (13) has been used to estimate the median lethal dose (ED_{50}) and its standard error (S.E.) by means of logarithmic-probit graph paper.

B. Results.—(*a*) *Rabbit. Single injection.*—Death occurred either within twenty minutes of the injection, probably due to ventricular fibrillation (11); or within six days due to nephrotoxic action (3). Table II summarizes results obtained following a single injection of 10% and 1% solutions of the mercurial diuretics.

Slow intravenous infusion.—Table III shows the average lethal dose in rabbits to be 27.4 mg./Kg. mercury for HMP and 55.1 mg./Kg. for Mersalyl-Theophylline.

(*b*) *Cat. Single injection.*—The results, summarized in Table II, show that in contrast to the rabbit, the mortality within ten days is lower with HMP than with the same dose of Mersalyl-Theophylline.

Slow intravenous infusion.—The average figure of toxicity of HMP is 40.5 mg./Kg. mercury; it is practically the same for Mersalyl-Theophylline

TABLE II.—MEDIAN LETHAL DOSE OF MERCURIAL DIURETICS FOLLOWING SINGLE INJECTION[a]

(Intravenous and Intramuscular Route)

Drug	Total No. of Animals Used I.V.	Total No. of Animals Used I.M.	Graphic Estimation ED_{50} I.V. ±S.E., Mg./Kg.	Graphic Estimation ED_{50} I.M. ±S.E., Mg./Kg.	Calculated LD_{50} Behrens I.V., Mg./Kg.	Calculated LD_{50} Behrens I.M., Mg./Kg.
			Rabbit			
Mersalyl-Theophylline	23	18	6.5±0.53	9.2±0.43	....	....
HMP	29	18	5.5±0.39	9.0±0.40	....	....
			Cat			
Mersalyl-Theophylline	15	30	8.0±1.08	19.5±3.10	10	22
HMP	18	27	17.8±1.72	30.0±2.70	23	33
			Rat			
Mersalyl-Theophylline	124	84	6.8±0.19	14.2±0.88	7.5	14
HMP	169	78	8.0±0.18	16.0±1.25	9.0	17

[a] Period of observation for the deaths recorded: 10 days.

TABLE III.—TOXICITY OF MERCURIAL DIURETICS

(Slow Perfusion in the Unanesthetized Rabbit)

Date of Assay	Weight of Rabbit	Rate of Perfusion, Mg./Kg./Min.	Duration of Experiment, Min.	Volume of Perfusion, Cc.	Lethal Dose, Mg./Kg.
		I. HMP (Recently Prepared)			
12/4/45	2.6	0.8	17	43	16
12/5/45	2.5	0.8	67	176	69
1/8/46	2.1	0.8	33	57	27
1/8/46	2.0	0.8	23	35	18
1/9/46	2.0	0.8	14	28	14
1/9/46	2.0	0.8	29	58	28
1/27/46	2.8	0.8	20	53	19
1/31/46	2.1	0.8	33	63	30
1/31/46	2.1	0.8	26	53	26
2/2/46	2.9	0.8	32	94	32
2/7/46	2.0	0.8	20	39	19
2/7/46	2.2	0.8	29	68	31
Average of 12 rabbits					27.4±7.7
		II. Mersalyl-Theophylline (Commercial Preparation)			
12/3/45	2.6	0.8	76	198	75
1/4/46	3.0	0.8	56	169	56
1/4/46	2.8	0.8	47	120	42
1/8/46	2.5	0.8	70	153	61
1/8/46	2.2	0.8	48	77	35
1/9/46	1.8	0.8	28	49	27
1/9/46	1.8	0.8	53	94	52
1/30/46	1.9	0.8	101	192	99
1/30/46	2.2	0.8	35	53	24
1/30/46	2.5	0.8	20	52	13
2/25/46	2.6	0.8	37	66	37
2/25/46	1.4	0.8	39	52	39
Average of 12 rabbits					55.1±4.2
$P_{[a]}$					0.01

[a] Probable coincidence calculated according to the method described by R. A. Fisher (12).

TABLE IV.—TOXICITY OF MERCURIAL DIURETICS
(Slow Perfusion in the Urethanized Cat)

Date of Preparation	Date of Assay	Weight of Cat, Kg.	Rate of Perfusion, Mg./Kg./Min.	Duration of Experiment, Min.	Volume of Perfusion, Cc.	Lethal Dose, Mg./Kg.
			I. HMP (Recently Prepared)			
9/19/45	9/19/45	2.80	1.08	29	113	40
	9/20/45	3.42	0.8	46	146	42
	9/20/45	3.94	0.8	45	176	44
	9/21/45	2.30	0.8	39	77	33
	9/24/45	3.64	0.8	50	178	49
9/25/45	9/25/45	2.52	0.8	30	70	30
	9/25/45	2.60	0.8	29	66	25
10/2/45	10/2/45	2.78	0.8	16	44	16
	10/2/45	2.90	0.8	56	164	56
	10/2/45	2.25	0.8	56	173	56
2/2/46	2/2/46	3.60	0.8	63	218	60
	2/2/46	2.54	0.8	36	113	32
Average of 12 cats						40.5±3.92
			II. Mersalyl-Theophylline (Commercial Preparation)			
	8/31/45	2.34	0.8	47	74	32
	9/4/45	2.74	0.8	43	138	49
	9/5/45	3.62	0.8	58	192	53
	9/6/45	3.54	0.8	44	158	45
	9/7/45	2.56	0.8	6	17	6
	9/7/45	2.06	0.8	28	69	27
	10/1/45	3.43	0.8	32	110	32
	10/3/45	3.18	0.8	39	105	38
	10/5/45	3.18	0.8	58	188	59
	10/5/45	1.80	0.8	44	83	46
	2/1/46	3.00	0.8	41	138	46
	2/4/46	2.20	0.8	103	228	103
Average of 12 cats						44.9±6.6
P^a						0.6

[a] Probable coincidence calculated according to the method described by R. A. Fisher (12).

TABLE V.—INFLUENCE OF AGING UPON THE TOXICITY OF HMP
(Slow Perfusion in the Urethanized Cat)

Date of Preparation	Date of Assay	Weight of Cat, Kg.	Rate of Perfusion, Mg./Kg./Min.	Duration of Experiment, Min.	Volume of Perfusion, Cc.	Lethal Dose, Mg./Kg.
6/13/45	9/10/45	2.30	0.8	57	13	5
	9/12/45	2.10	0.8	15	31	15
	9/12/45	2.35	0.8	47	157	47
	9/14/45	3.14	0.8	38	118	37
	9/14/45	2.78	0.8	34	93	34
	9/14/45	3.46	0.8	38	127	37
	9/14/45	3.10	0.8	48	144	46
Average of 7 cats						38.9±5.0

TABLE VI.—COMPARATIVE TOXICITY AND DIURETIC ACTIVITY OF MERCURIALS

Animal Species	Rabbit		Cat		Rat	
Drugs	Mersalyl-Theophylline	HMP	Mersalyl-Theophylline	HMP	Mersalyl-Theophylline	HMP
Toxicity Ratio Routes $\frac{ED_{50}\text{ Intravenous}}{ED_{50}\text{ Intramuscular}}$	0.7	0.6	0.41	0.59	0.47	0.50
Toxicity Ratio Drugs $\frac{ED_{50}\text{ Mersalyl I.V.}}{ED_{50}\text{ HMP I.V.}}$	0.85	1	2.2	1	1.18	1
$\frac{ED_{50}\text{ Mersalyl I.M.}}{ED_{50}\text{ HMP I.M.}}$	0.97	1	1.5	1	1.12	1
Average Effective Diuretic Dose	1.98	2.34	5.94	9.26	7.92	9.30
Safety Margin Intramuscular	4.60	3.85	3.29	3.30	1.80	1.50

(44.9 mg./Kg. mercury, Table IV). Although there are fairly wide variations between individual animals in the 2 groups, it is interesting to note that the results closely approximate those reported by Modell and Krop (4), who obtained a value of 36.3 mg./Kg. mercury for Mersalyl-Theophylline, injected at the same rate of 0.8 mg. mercury/Kg.

Influence of Aging.—Modell and Krop (4) found that the toxicity of Mersalyl-Theophylline solutions decreased when allowed to stand for a period of one month at room temperature. These results were assumed to be due to some form of chemical reaction between the components. A similar experiment was made with HMP after it had aged three months. As reported in Table V, no significant variation of toxicity was noticed; the average lethal dose determined by slow infusion in 7 cats being 38.9 mg. mercury/Kg.

(*c*) *Rat. Single injection.*—The percentage of mortality at doses varying between 5.5 mg./Kg. and 30 mg./Kg. of mercury is summarized in Table II. It appears from this table that the toxicity of the 2 compounds is quite similar. Although the number of animals treated was not very large, a comparison of the toxicity of 0.5, 1, and 2% solutions shows that a variation in concentration did not influence the lethal effect of the drug. This observation confirms results obtained with Mersalyl-Theophylline by Modell and Krop (4).

(2) **Intramuscular Toxicity.**—A study of the acute toxicity by the intramuscular route has also been made in 3 animal species: the rabbit, the cat, and the rat.

A. Material and Method.—One hundred sixty albino male rats, weighing between 125 and 150 Gm.; 56 male cats, weighing between 1.5 and 2.5 Kg.; and 36 male albino rabbits, averaging 2 Kg. in weight, were used. The solution of each drug (*p*H 8.1 for HMP and 8.9 for Mersalyl-Theophylline) was injected without further dilution in the posterior tibiales muscle and the animals observed during a period of ten days.

B. Results.—Data are presented in Table II. As previously observed by the intravenous route, an individual variation of susceptibility toward animal species is to be noted. HMP appears to be less toxic than Mersalyl-Theophylline in the cat; whereas, the toxicity of both drugs is about the same in the rabbit and the rat.

Table VI summarizes a comparison of the ED_{50} values for various species and routes of administration. The ratio ED_{50} Intravenous/ED_{50} Intramuscular is about the same for both compounds in the rat and rabbit; whereas, it is somewhat higher for HMP than for Mersalyl-Theophylline in the cat. A comparison of the relative toxicity of HMP and Mersalyl-Theophylline administered by the intravenous route gives very similar data in the rabbit and in the rat, but in the cat, HMP appears to be about twice as toxic as Mersalyl-Theophylline. The relative toxicity of the 2 drugs following intramuscular injection is about 1 in the rabbit and the rat, but 1.5 in the cat. The safety margin by the intramuscular route, however, remains approximately the same for HMP and Mersalyl-Theophylline in the 3 animal species: 3.85 and 4.60 in the rabbit; 3.30 and 3.29 in the cat; 1.5 and 1.8 in the rat.

DISCUSSION

A quantitative comparison of the diuretic activity of HMP and Mersalyl-Theophylline administered intramuscularly in equal doses to rats and rabbits shows that these 2 compounds have nearly the same diuretic effect. The diuretic ratio in the rat is 1.31 for Mersalyl-Theophylline and 1.25 for HMP; 3.25 for Mersalyl-Theophylline and 3.00 for HMP in the rabbit, figures which may be considered to fall within the limits of experimental error. In the cat, however, HMP shows 30% less activity, the diuretic ratios being 3.65 and 5.27, respectively. It should be emphasized, however, that although the experimental method is considered to be very reliable, an unequivocal answer concerning the usefulness of a diuretic can only be given by a study of the *relief of edema in man* (8).

As far as *intravenous toxicity* is concerned, the results vary according to the animal species used; in cats and rats, HMP appears to be less toxic than Mersalyl-Theophylline, whereas in rabbits, HMP is more toxic. The possibility has to be considered, however, that such variation might depend upon the rate of administration and the concentration of the drug rather than upon the animal species. As a matter of fact, in cats, in the case of slow infusion a 0.2% solution is used; while, by single injection in cats, more concentrated solutions (1% to 10%) are rapidly injected at once. The factor of concentration is most important, indeed, because, if a mercurial is introduced rapidly at a high concentration into the blood stream, it may suddenly induce ventricular fibrillation (3, 10, 11). This toxic effect does not occur when a diluted solution is administered slowly.

Experiments made by injecting intravenously in cats a concentrated solution (10%) of mercurial show that the toxicities of Mersalyl-Theophylline and HMP are nearly the same in this species. On the other hand, results obtained by slow infusion of a 0.5% solution in the rabbit prove that HMP is more toxic than Mersalyl-Theophylline as revealed by a comparison of the toxicity ratio—rapid single injection/slow continuous infusion. Moreover, a comparison in the same animal species (rabbit or rat) of the toxicity values obtained with different concentrations of HMP (0.5%, 1%, 2% in the rat; 1% and 10% in the rabbit) injected by the same technique (single injection) reveals no variation in the lethal dose provided the solution is injected slowly and regularly. Thus it is obvious that the difference in toxicity as revealed by different methods is due neither to the rate of injection nor to the concentration of the solution, but to the difference in sensitivity of the animal species.

If the route of administration is changed from intravenous to intramuscular, the toxicity is decreased in the 3 animal species investigated. An interesting fact to be noted is that following intramuscular injection in the cat, HMP is less toxic than Mersalyl-Theophylline, whereas the toxicity is about the same for the 2 compounds administered by this route in the rat and the rabbit.

Aging has no influence upon the toxicity of the HMP-Theophylline mixture, no decrease being observed after standing for three months. This fact is in contrast with the data reported in the literature for Mersalyl-Theophylline (4).

A comparison of the average effective diuretic dose and the toxic dose, both measured by intramuscular route, shows that the safety margin of the 2 drugs remains about the same in the rat, the rabbit, and the cat.

CONCLUSIONS

1. Sodium N - (β - hydroxymercuri - γ-methoxypropyl) - 2 - pyridone - 5 - carboxylate in combination with Theophylline has about the same diuretic activity in the rat and the rabbit as Mersalyl-Theophylline; it has been found to be less active, however, in the cat.

2. In the rabbit, either by single intravenous injection or by slow continuous infusion of 1 and 10 per cent solutions, HMP is more toxic than the same dose of Mersalyl-Theophylline. By intramuscular injection, on the contrary, the toxicity of both compounds is approximately the same.

3. In the cat, by slow continuous infusion, Sodium N-(β-hydroxymercuri-γ-methoxypropyl) - 2 - pyridone - 5 - carboxylate has approximately the same toxicity as Mersalyl-Theophylline. By single intravenous injection and by the intramuscular route HMP is less toxic than Mersalyl-Theophylline.

4. In the rat, either by single intravenous injection or by intramuscular injection, the HMP and Mersalyl-Theophylline have about the same toxicity.

5. In the cat, no detectable change in intravenous toxicity seemed to occur in solutions of HMP allowed to stand for three months at room temperature.

6. The safety margin of the 2 compounds following intramuscular injection is about the same in the 3 animal species investigated.

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Further Studies on Two New Lysergic Acid Compounds: *d*-Lysergic Acid-*dl*-Hydroxybutylamide-2 (Methergine) and Dihydroergotamine (D.H.E. 45)*,†

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The results of a study involving a comparison of the systemic effects of temperature, respiration, and pupil size in rabbits for methergine, dihydroergotamine, and ergonovine are reported.

RECENTLY, two newer ergot alkaloids have received considerable clinical trial in this country. Both drugs are partly synthesized from a lysergic acid radical with changes in the side chains but possess different pharmacologic actions. The compound called Methergine[1] (1) is *d*-lysergic acid *dl*-hydroxybutylamide which is similar in chemical structure to Ergonovine but has one more methylene group. It has a potent oxytocic action and, already, its clinical use in obstetrics has resulted in several favorable reports. The other compound introduced under the experimental number of D.H.E. 45 (2) is Dihydroergotamine.[1] Its chemical structure is similar to Ergotamine but it is claimed to have a less toxic action and to be a stronger sympathicolytic agent than the older drug. Several recent reports point out its effectiveness in the treatment of migraine and other sympathicotonic disorders.

METHERGINE

In 1944 we (3) reported on some of the pharmacologic actions of Methergine and its use in 22 obstetrical cases where the drug was given intravenously in a dose of 0.2 mg. at the time of the birth of the anterior shoulder. Used in this manner, Methergine hastened the delivery of the placenta, decreased the blood loss postpartum, and surpassed Ergonovine in duration and intensity of action. Since then several additional reports on the use of this drug in labor have appeared. Table I presents a summary of the results obtained by the various authors who have reported on Methergine (4).

TABLE I.—SUMMARY OF REPORTS ON INTRAVENOUS USE OF METHERGINE DURING THE THIRD STAGE OF LABOR

Authors	Year	No. of Cases	When Drug Given	Speed of Action, Sec.	Duration 3rd Stg., Min.	Average Blood Loss Meth., Cc.	Average Blood Loss Control, Cc.	Comparison with Ergonovine
Janke	1942	1667	B.A.S.[a]	..	...	....	...	Less blood loss
Kirchhof, Racely, Wilson, David	1944	22	B.A.S.	..	3.2	150	..	Equal to Ergonovine
Roberts	1944	34	A.B.B.[b]	30	2–5	181	247	Rock-like firmness fundus for longer time
Tollefson	1944	200	S.P.S.[c]	48	..	98	..	Suggests drug be given after placenta separation
Brougher	1945	271	B.A.S.	30	...	50–100	...	Recommends fundus massage at time drug given
Cartwright and Rogers	1946	252	B.A.S.	..	3.4	Less	...	As effective and safe as Ergonovine
Tritsch and Schneider	1945	120	B.A.S.	..	Less	Less	...	Equal to Ergonovine

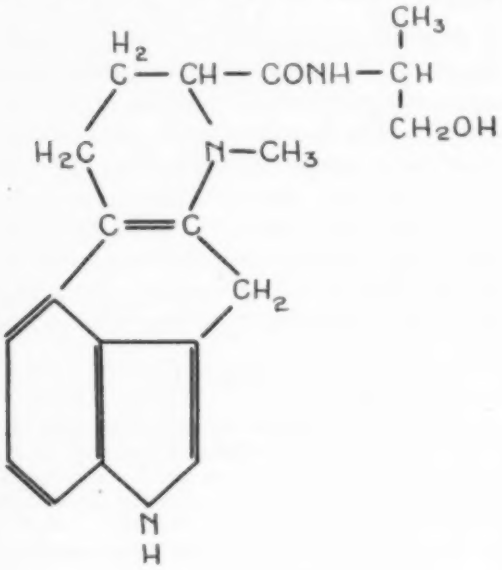
[a] B.A.S.—Birth of anterior shoulder.
[b] A.B.B.—After birth of baby.
[c] S.P.S.—Start of placental separation.

The current report of Farber (5) on the use of this new oxytocic for the safe induction of labor in 30 cases is of interest. In Farber's first 11 cases, 5

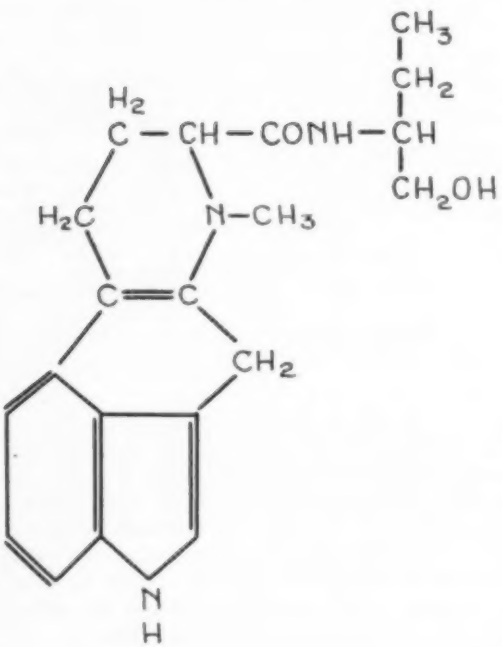
* Received Sept. 4, 1946, from the Department of Pharmacology, University of Oregon Medical School, Portland, Ore.
Presented to the Scientific Section, A. PH. A., Pittsburgh meeting, 1946.
† Supported in part by the Research Council, Oregon State Board of Higher Education, Corvallis, Ore., and the Sandoz Chemical Works, Inc., New York, N. Y.
‡ We wish to thank Mr. Harry Althouse, Mgr., West Coast Branch, Sandoz Chemical Works, Inc., San Francisco, and Dr. K. K. Chen, Eli Lilly Research Laboratories, Indianapolis, Ind., and Mr. C. B. Groshong, Eli Lilly Representative, Portland, Ore., for furnishing liberal supplies of Methergine, Dihydroergotamine (Sandoz) and Ergotrate (Ergonovine Maleate), and Sodium Pentobarbital (Eli Lilly).
[1] Both Methergine and Dihydroergotamine are products of Sandoz Chemical Works, Inc., New York, N. Y.

minim doses of Methergine were given orally every hour for five doses with successful induction of labor in 8 cases. His next 19 cases were given a dose of 1.0 cc. of Methergine every hour for five times with successful induction in each patient and less blood loss during the third stage than in those going into labor spontaneously.

The introduction of Methergine represents a significant advance in the chemistry of ergot alkaloids because it is the first alkaloid of this group to be made partly by synthesis and to depend entirely on this process for its manufacture. Stoll and Hofmann (1) condensed isolysergic acid azide with *d*-2-amino-1-butanol, subjected the product thus formed to a transposition treatment and obtained *d*-lysergic acid *dl*-hydroxybutylamide 2 which was given the laboratory number of M.E. 277 and, later, Methergine. The resultant compound differs structurally from Ergonovine in possessing an extra CH_2 group in the alcohol amide side chain as shown in Fig. 1.



ERGONOVINE



METHERGINE

Fig. 1.

Pharmacologically, in studying the action of Methergine on the uterus we found that it differed very slightly from Ergonovine with respect to the speed, intensity, and duration of effect. Equal dilutions of either drug tested on isolated strips of guinea-pig or rabbit uterus suspended in Locke's solution gave similar motor responses.

Intravenous administration of either Methergine or Ergonovine in the barbitalized dog usually gave a slight increase in blood pressure and enhanced the effect of subsequent injections of epinephrine. Likewise, since it was found that both these drugs failed to inhibit active motor contractions of the rabbit uterus caused by epinephrine, it appears that neither Ergonovine nor Methergine are adrenolytic.

DIHYDROERGOTAMINE

Dihydroergotamine was first patented by May (2) who hydrogenated the readily reducible double bond of lysergic acid and obtained a well-defined crystallized dihydrogenated compound. A tentative, incomplete formula of this new compound and the accepted one for Ergotamine (6)[1] are shown below.

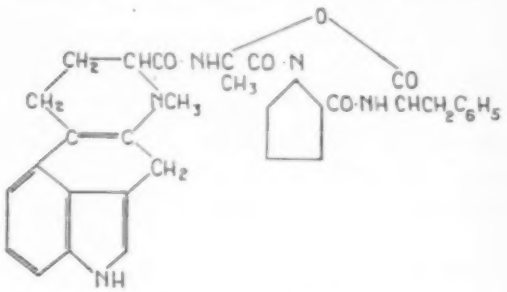


Fig. 2.—Ergotamine.

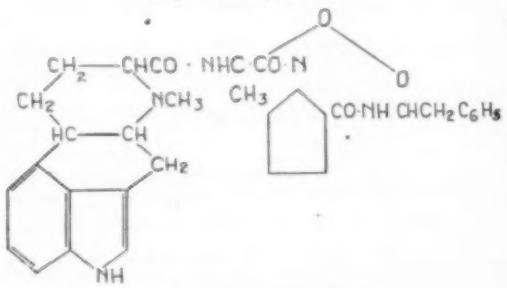


Fig. 3.—Dihydroergotamine.

[1] In a later publication Craig, Shedlovsky, Gould, and Jacobs [*J. Biol. Chem.*, 125, 289(1938)] place the carboxy group in lysergic acid on position 8 instead of position 7, as shown in Figs. 2 and 3. Thus the carboxy group should be attached to the carbon atom located in the upper left-hand part of the structural formula where the CH_2 is now placed, for both Ergotamine and Dihydroergotamine.

Preliminary investigations on dihydroergotamine by Rothlin and Bruegger (7) showed that this, and two other dihydrogenated ergot alkaloids, compared with the natural alkaloids Ergotamine and Ergotoxine, possessed less toxicity for small animals, were less emetic, showed no effect in exciting the rabbit uterus, and reduced arterial pressure and slowed the pulse on intravenous administration to the narcotized dog. Its sympathicolytic or adrenalin-inhibitory effect on the isolated rabbit uterus was found to be five times greater than that of ergotamine. This evidence indicated that this new drug should serve as an effective substitute for Ergotamine in the treatment of migraine, herpes, angiospastic conditions, and other sympathicotonic disorders. Accordingly, it has been used successfully in these conditions by a number of workers (8).

In our paper on methergine (3) we included for the purpose of comparing the effects of Methergine and D.H.E. #45 on the blood pressure in the anesthetized dog a figure demonstrating the sympathicotytic effect of D.H.E. 45 when subsequent injections of epinephrine were given. We used intravenous Sodium Pentobarbital for anesthesia in these dogs. Recently, however, Orth (9) has reported that D.H.E. 45 injected in large doses of 0.4 mg./Kg. "does not produce an epinephrine-reversal of the blood pressure in dogs anesthetized with nembutal, amytal, ether or chloroform." Instead, in direct contrast to the observations we and others have made, Orth found an elevation of the blood pressure when subsequent doses of epinephrine are injected after D.H.E. 45. Koppanyi (1941) (10) has shown that Ergotamine fails to reverse the pressor effects of epinephrine in dogs and cats deeply anesthetized with barbiturates. Orth also showed that D.H.E. 45 lacks the usual Ergotamine oxytocic effect by giving the drug daily to rats during the entire gestation period and noting that normal litters were born and nurtured successfully while the injections continued. He also found, that, unlike Ergotamine Tartrate, gangrene of the rat's tail was rarely produced by D.H.E. 45 even after doses were given for more than thirty days.

In addition to having certain pharmacologic actions, although each of a different type, on such structures as the uterus, intestines, heart, and blood vessels, Ergonovine and Ergotamine both influence the body metabolism and the iris of the eye in the rabbit. de Beer and Tullar (11) have studied the pyrexic and mydriatic effect produced by Ergonovine in rabbits and have proposed it as a method for the biological standardization of this drug based on the straight-line logometric curves plotted from the quantitative response-dose results obtained. White (12) has recently reviewed the literature on the influence of ergot drugs on temperature of experimental animals.

In continuing our investigation of the pharmacology of Methergine and Dihydroergotamine we have undertaken to extend the series in our preliminary observations on these drugs by a further study of the systemic effects of temperature, respiration, and pupil size. Ergonovine has been used in the same manner for comparison.

METHODS

Sixteen rabbits each were used for the study of each drug. Methergine, Dihydroergotamine, or Ergonovine was given intravenously to groups of 4 rabbits in each of the following doses: 0.4 mg., 0.8 mg., 1.2 mg., and 1.6 mg. per Kg. In addition, four control rabbits were strapped on animal boards and exposed under identical conditions and for the same length of time after placebo injections of 1.0 cc. of saline. Records were kept of the rectal temperature and the pupil size was measured. On one or two animals in each group sample kymograph records of the respiratory rate were made. All of these experiments were done at night, under constant conditions of room temperature and with a constant amount of artificial light. Two rabbits, in addition to the others, were given local instillation of 10 drops of Methergine in one eye and observed for pupillary enlargement.

For the human trial of Methergine, 5 normal medical students were given an intravenous dose of 0.2 mg. of the drug while 5 other students received a placebo of 1.0 cc. of physiological saline to serve as controls. Frequent records were made of their pulse, blood pressure, temperature, respiratory rate, and their subjective symptoms noted for one hour after injecting the drug. Similarly, 5 normal medi-

TABLE II.—EFFECTS OF METHERGINE AND ERGONOVINE ON THE TEMPERATURE AND PUPIL SIZE WHEN GIVEN INTRAVENOUSLY TO RABBITS

Dose, Mg./Kg.	Temperature Increase		Increase in Pupil Size	
	Methergine, °F.	Ergonovine, °F.	Methergine, Mm.	Ergonovine, Mm.
0.4	0.2	−0.8	2.5	0.0
	0.5	3.0	1.5	2.0
	2.5	3.5	1.5	3.0
	0.0	0.6	2.5	0.0
	Av. 0.8	1.6	2.0	1.2
0.8	0.7	2.9	4.5	4.5
	2.4	4.7	3.0	3.5
	1.6	3.4	4.0	5.0
	0.5	0.0	3.5	3.5
	Av. 1.3	2.7	3.7	4.1
1.2	1.8	1.7	4.5	5.5
	4.9	3.7	2.5	3.5
	2.0	4.0	5.0	4.0
	1.4	1.8	6.0	5.0
	Av. 2.5	2.8	4.5	4.5
1.6	3.1	5.0	3.0	4.0
	5.6	5.6	3.0	4.5
	4 2	..[b]	5.5	..[b]
	5.2[a]	..[c]	3.0	4.0
	..	1.6	..	2.0
	Av. 4.5	3.5	3.6	3.6

[a] Methergine rabbit died 2 hr. after drug in convulsions.
[b] Ergonovine rabbit died immediately after injection in convulsions.
[c] Ergonovine rabbit died 45 min. after injection in tetanic convulsions.

cal students received Ergonovine Tartrate in a dosage of 0.2 mg. and were observed in the same manner.

RESULTS

The results for the intravenous injection of increasing doses of Methergine and Ergonovine are summarized in Table II. Table III presents the results for the injection of Dihydroergotamine in rabbits. The analysis of the temperature and pupillary changes recorded for the rabbits given D.H.E. 45 were made in a different manner than for the Methergine-Ergonovine group since all these animals were found to have a decrease in temperature following injection. Four control rabbits, observed at the same time, were analyzed in this same manner for comparison. The two rabbits receiving local instillations of Methergine in the eye showed no pupillary changes. Nor did the medical students given intravenous injections of Ergonovine, Methergine, and placebos show any significant differences in any of the various observations made.

TABLE III.—EFFECTS OF INTRAVENOUS INJECTIONS OF D.H.E. 45 ON THE TEMPERATURE AND PUPILS OF RABBITS (AVERAGE)

Dose, Mg./Kg.	No. Rabbits Used	Temperature Changes,- °F. Av.	Pupillary Changes,[a] Mm.
0.4	4	−0.7	0
0.8	4	−0.37	0
1.2	4	−0.27	0
1.6	4	−0.7	+0.2
Controls	4	0.05	+0.5

[a] Temperature and pupillary changes are differences in readings immediately before injection and ½ hr. afterward Control averages were obtained in the same manner.

DISCUSSION AND CONCLUSIONS

Hyperpyrexia and mydriasis from the use of Methergine in humans during labor will probably never be noticed because of the small dose required for therapeutic effects compared with the doses we used in rabbits. The recommended therapeutic dose of Methergine is 0.2 mg. intravenously or intramuscularly when used postpartum while we administered to one group of rabbits an amount equivalent to 8 times this dose per each kilogram of weight. Examination of Table II shows that both Methergine and Ergonovine, in equivalent dosage, produce about the same degree of changes in these animals. On the other hand, as shown in Table III, Dihydroergotamine injection into rabbits results in just the opposite effects with a general decrease in temperature more than that noted for the control rabbits and with very little change in pupil size. When the highest dosage of Ergonovine (1.6 mg./Kg.) was used, 2 of 5 animals died in convulsions while 1 of 4 rabbits given the same dose of Methergine died. None of the Dihydroergotamine rabbits died. From these data it might be judged that when information is available for the acute toxicity of Methergine and Dihydroergotamine, these two new compounds will be found to have a high therapeutic index. While a lower degree of hyperpyrexia is shown for Ergonovine (3.5° F., av.) than for Methergine when given a dose of 1.6 mg./Kg., the fact that data from only 3 animals surviving Ergonovine injection were averaged accounts for this statistical variation. The increase in respiratory rate generally observed following drug administration was in all probability an accompanying phenomena of pyrexia, although a direct action of these drugs on the respiratory center cannot be ruled out by our observations.

In summary, these results show the significant fact that lysergic acid, when coupled with different type side chains, will have entirely different effects as shown by the differences observed for Methergine and Dihydroergotamine. The lysergic acid-alcohol amide group, such as Ergonovine or Methergine, is markedly utero-active and, as shown by these experiments, has similar systemic effects in rabbits. On the other hand, the lysergic-acid complex chain type of compound, such as Ergotamine, may have its degree of utero-activity changed by saturation of the double bond (Fig. 3) in lysergic acid (as in Dihydroergotamine) and, although still a lysergic acid compound, show a completely different systemic effect from the lysergic acid-alcohol amide compounds. Thus, as studies progress we can look for the introduction of better and more ideal ergot drugs and, perhaps, an eventual solution to the age-old Ergot Problem.

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A Study of Antacid Buffers: I. The Time Factor in Neutralization of Gastric Acidity*

By J. M. HOLBERT, NANCY NOBLE, and I. W. GROTE

The U. S. P. XII test for measurement of the acid consuming capacity of aluminum hydroxide gel and dried powder does not take into account the time required for acid neutralization, being simply a measure of excess acid neutralized after 1 hour. Since the patient using such tablets is perhaps equally interested in the rapidity of neutralization, a test has been devised in which a weighed amount of the antacid buffer is treated with artificial gastric juice, and the *p*H rise measured at the end of 1, 5, 10, etc., minutes. Results are given when the test is applied to various types of liquid and dried aluminum hydroxide and similar types of buffers.

THE U. S. P. XII test for determining the acid consuming capacity of aluminum hydroxide gel and dried aluminum hydroxide gel does not take into account the time required for acid neutralization, but is a measure of excess acid neutralized after one hour at 37.5°. Since the relief of pain owing to excess gastric acidity is probably a function of the change in *p*H of the gastric contents, the patient is perhaps more interested in the rapidity of neutralization than in the total amount of acid that can be neutralized over a period of one hour.

According to U. S. P. XII, p. 200, the test for the acid consuming capacity (a. c. p.) of dried aluminum hydroxide gel is to add 100 cc. 0.1 *N* HCl to a 0.25-Gm. sample of the material to be tested and to agitate continuously at 37.5° for one hour. At the end of this time, the excess acid is back titrated with 0.1 *N* NaOH using bromphenol blue T. S. as an indicator. The procedure for the gel is similar except that a 1.5-cc. sample is treated with 150 cc. of 0.1 *N* HCl. The results are then expressed as the number of cc. of 0.1 *N* HCl neutralized by 1 Gm. of the material. This value must fall between certain limits.

EXPERIMENTAL

It is our concept that while this method is quite adequate for determining the neutralizing or acid consuming power of a material, it does not give a reliable therapeutic value as an antacid. In support of this statement, tests on three well-known antacids were run. In all cases the directions given in the U. S. P. were followed except that the *p*H of the mixture was determined at various intervals. The results are given in Table I.

The change of *p*H under these conditions is so slight, owing to the excess of acid used, that the materials would appear worthless as far as the relief of gastric pain is concerned, yet each of the above products enjoys wide use as an antacid. Thus the mere neutralization of excess hydrochloric acid after a one-hour period is not a sufficient datum on which to evaluate the therapeutic value of an antacid.

Although this test should be retained, in addition, a test should be added that more closely simulates conditions present in cases of hyperacidity and which should be designed to measure the change of *p*H that occurs after addition of the antacid. Since the time element is of paramount importance to the patient, this factor should also be considered. For some time now in our own work we have been using

* Received Sept. 4, 1946, from the Brayten Pharmaceutical Company, Chattanooga, Tenn.
Presented to the Scientific Section, A. PH. A., Pittsburgh meeting, 1946.

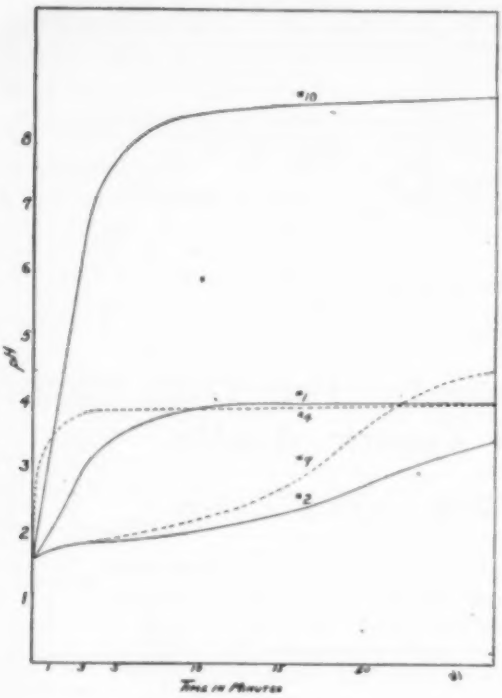

Figure 1

a procedure to test antacids which combines these features. The results, we believe, allow a better interpretation of the therapeutic value of antacids.

In the actual procedure a one-gram sample of finely ground tablet material is added to 150 cc. of artificial gastric juice maintained at 37.5° (1). The mixture is mechanically agitated. The *p*H of of the mixture is determined at regular intervals with a Beckman *p*H meter. Usually the test is run for only thirty minutes since this seems ample time for a patient to wait for relief.

The choice of an artificial gastric juice rather than 0.1 *N* HCl is self evident; but there exists the need of a standard artificial gastric juice. The volume of gastric juice was chosen as 150 cc. because it seems a fair approximation of the amount of gastric juice present in the resting stomach (2). The sample weight of 1 Gm. was taken since most antacid tablets approximate one gram in weight. Observation of *p*H at regular intervals is necessary to determine how fast and how well the antacid is neutralizing the excess acid. The results from a series of these tests are given in Table II.

Figure 1 gives a graphic illustration of some of the results from Table III.

According to Johnson and Duncan (3) an antacid should be mildly alkaline, promptly neutralizing

TABLE I

Time, Min.	Aluminum Hydroxide Tablets *p*H	Aluminum Hydroxide Tablets A. C. P.	Aluminum Dihydroxy Aminoacetate Tablets *p*H	Aluminum Dihydroxy Aminoacetate Tablets A. C. P.	Aluminum Hydroxide Magma *p*H	Aluminum Hydroxide Magma A. C. P.
0	0.99	....	0.99	....	1.08	....
1	1.00	....	1.08	....	1.09	....
3	1.01	....	1.22	....	1.10	....
5	1.03	....	1.24	....	1.10	....
10	1.12	....	1.27	....	1.10	....
15	1.21	....	1.27	....	1.10	....
20	1.28	....	1.27	....	1.10	....
30	1.34	....	1.27	....	1.10	....
40	1.36	....	1.27	....	1.10	....
50	1.36	....	1.27	....	1.10	....
60	1.36	230 cc. 0.1 *N* HCl per Gm.	1.27	136 cc. 0.1 *N* HCl per Gm.	1.10	16.95 cc. 0.1 *N* HCl per Gm.

TABLE II[a]

Time, Min.	*p*H 1	2	3	4	5	6	7	8	9	10	11	12
0	1.60	1.58	1.56	1.53	1.55	1.56	1.56	1.59	1.60	1.56	1.56	1.66
1	2.00	1.73	1.59	2.17	3.42	1.65	1.70	1.69	5.20	3.78	2.20	6.50
3	2.90	1.81	1.68	3.48	3.80	1.83	1.80	2.10	5.64	6.28	5.40	6.55
5	3.49	1.86	1.78	3.80	3.85	1.97	1.90	2.38	5.85	7.72	5.95	6.62
10	3.89	2.01	2.05	3.91	3.92	2.23	2.20	3.62	6.08	8.41	6.40	6.78
15	3.96	2.26	2.22	3.93	3.93	2.46	2.55	4.47	6.26	8.52	6.85	6.92
20	3.98	2.70	2.41	3.94	3.95	2.59	3.62	4.99	6.43	8.61	7.40	7.06
30	3.99	3.46	2.72	3.96	3.98	2.78	4.47	5.43	6.77	8.70	8.06	7.38

[a] Key:
1—Aluminum dihydroxy aminoacetate.
2—Aluminum hydroxide, Brand A.
3—Aluminum hydroxide, Brand B.
4—Aluminum hydroxide magma, Brand A, 2t.
5—Aluminum hydroxide magma, Brand B, 3t.
6—Aluminum hydroxide + magnesium trisilicate.
7—Magnesium Trisilicate.
8—Magnesium trisilicate + calcium carbonate.
9—Magnesium trisilicate + calcium carbonate + magnesium carbonate.
10—Magnesium hydroxide.
11—Magnesium hydroxide + calcium carbonate + bismuth subcarbonate + sodium bicarbonate.
12—Sodium bicarbonate.

free hydrochloric acid to a *p*H of 3.5 to 4. Although pepsin is active from *p*H 1 to 7, it has been repeatedly demonstrated that most of its activity stops at *p*H 3.5. From the foregoing table it is readily seen that while some antacids reach this *p*H rapidly, others may require 30 minutes or more. The test also serves to point out those nonbuffering antacids that are alkalizing agents and may lead to acid rebound (4). A novel approach to the antacid problem was suggested by Martin and Wilkinson (5) who used finely divided resins to adsorb H^+ ion and inactive pepsin.

Since some antacid tablets are to be swallowed whole and not chewed it seemed desirable to conduct a few tests using whole tablets. The tablet was suspended in a small wire basket which was raised and lowered through the acid solution in such a fashion as to simulate peristaltic action of the stomach. The results from this modification are given in Table III.

TABLE III

Time, Min.	*p*H Aluminum Dihydroxy Aminoacetate Tablet	*p*H Aluminum Hydroxide Tablet, Brand A	*p*H Aluminum Hydroxide Tablet, Brand B
0	1.49	1.50	1.50
1	1.60	1.54	1.47
3	2.48	1.61	1.49
5	3.12	1.64	1.52
10	3.66	1.78	1.68
15	3.78	1.90	1.83
20	3.79	2.10	2.02
30	3.80	2.93	2.38

Here the neutralizing action is not as rapid as with the powder but the same general order of neutralization rate is obtained.

After the laboratory work for this paper was completed, an article by the English workers, E. H. Johnson and J. Duncan (3), came to our attention. They also point out the need of a time evaluation. They first ran a preliminary test which measures the amount of the antacid necessary to raise 100 cc. of 0.1 *N* HCl at 37° to *p*H 3.5 in fifteen minutes. This they call the equivalent dose of the antacid. Next they add twice the equivalent dose of the antacid to 100 cc. of 0.1 *N* HCl at 37° and take the *p*H at ten-minute intervals. At each interval 20 cc. of the total volume is removed to simulate partial emptying of the stomach and 20 cc. of 0.1 *N* HCl is added to act as fresh acid from the stomach until the antacid fails to raise the *p*H of the total volume to 3.5. Under these conditions of test, with magnesium oxide the mixture goes immediately to *p*H 9.5 and continues to raise fresh 20-cc. portions to *p*H 3.5 for forty-five minutes, while sodium bicarbonate and calcium carbonate each raise the *p*H of the mixture to 7.5 and each has antacid power for fifty minutes. Magnesium trisilicate goes to *p*H 6.0 and holds for only forty minutes. A good grade of aluminum hydroxide goes to *p*H 3.5 only but will hold this value for a total of eighty minutes, which the consensus agrees is the preferable behavior of an antacid.

SUMMARY

1. The present U. S. P. neutralization test for antacids is inadequate since the time factor required for neutralization is ignored.

2. A new test is proposed which takes into account both the extent of *p*H change and time required for acid neutralization.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

1-Phenyl-2-aminoethanol
Dehydrothiotoluidine
6-Amino-2-phenylbenzothiazole
5-Amino-2-phenylbenzoxazole
1-Glyceraldehyde
Orcinol
p-Acetoxymercuriacetanilide
Carnosine
Parasorbic acid
Squalene
Synthaverin
Octaverin
Perparine
Hyaluronic acid
Desoxyribose
Phytic acid
1-Fluoro-2-hydroxyethane
dl-Erythronic acid
d-Glucurone
Laudanidine

The Absorption and Excretion of the Solid Polyethylene Glycols ("Carbowax" Compounds)*

By C. BOYD SHAFFER and FRANCES H. CRITCHFIELD†

The extent of gastrointestinal absorption of several solid polyethylene glycols including those designated as "Carbowax" compounds 1000, 1540, 4000, and 6000 has been determined following their administration to rats and to humans. In rats no absorption of the two higher molecular weight compounds was noted and with the two lower molecular weight compounds absorption was slight. When administered intravenously to human subjects "Carbowax" compounds 6000 and 1000 were excreted readily and to a high degree.

THE QUESTION of the gastrointestinal absorption of the solid polyethylene glycols is one upon which direct information has been lacking since these substances came under toxicological observation several years ago. It is obvious that exact knowledge on this point had to await the development of a quantitative analytical method, but even qualitative investigations were impossible because there were not methods available for the identification of these compounds. Recently, however, with the discovery of the reaction between the polyethylene glycols and certain complex inorganic acids, a method for the quantitative determination of the former has been developed (1), and this method has enabled the following investigation of the intestinal absorption of these materials in the rat and man.

The polyoxyethylene glycols dealt with in this report are a series of four solids well known in the pharmaceutical industry under the trade-mark name of "Carbowax" Compounds.[1] Physically, they are wax-like substances of varying degrees of hardness, and are soluble in water, aromatic hydrocarbons, acetone, and ethyl alcohol. Each is actually a mixture of several polymers, the average molecular weight of which is appended to the trade-name as a means of distinguishing the individual members of the series. Included in this group are "Carbowax" Compounds 1000, 1540, 4000, and 6000. A "Carbowax" Compound 1500 is also available, but it is in reality a blend of 1540 with a liquid polyglycol and hence has been omitted from this discussion.

In animal experimentation a number of satisfactory procedures are available for the study of intestinal absorption. Preference has been given to the method devised by Cori (2) as it allows quantitative estimation of the absorbing capacity of the whole intestine as a physiological unit and permits expression of the results in terms of body weight. With human subjects the approach is of necessity less direct, and in these instances we have followed the scheme developed by McCance and Madders (3) in their studies of the absorption of sugars. They arrived at an estimate of intestinal absorption by a comparison of the rate and amount of each sugar excreted (*a*) where injected intravenously, and (*b*) where taken by mouth. The polyglycols appeared to fit the pattern of such an approach as they are all excreted readily by the kidney and are only comparatively slowly destroyed in the tissues. Extensive animal experimentation over a period of several years had convinced us that at least relatively small amounts could be injected intravenously with safety, and that considerably larger amounts could be taken by mouth with no ill effects. The experience of the present work has confirmed that expectation in that eighteen separate intravenous injections of 1 Gm. each and fifteen oral doses of 10 Gm. each have been given to six adult male subjects with no untoward reactions in any case.

EXPERIMENTAL

Experiments with Rats.—Two hundred and twelve animals were used in this phase of the investigation. The procedure followed Cori's description in every respect, including the performance of the various

* Received Nov. 9, 1946, from Mellon Institute of Industrial Research, Pittsburgh, Pa.

† The authors are indebted to Carrol S. Weil for statistical analysis of the data presented herein and to Charles A. Walker for assistance in performance of the analytical work.

[1] Carbide and Carbon Chemicals Corporation.

control experiments listed by him as necessary checks upon the accuracy of the different steps involved. The analytical work was done according to the gravimetric method described in a preceding paper (1), with the exception that aliquots of the intestinal washings were clarified by the addition of one part of 10% zinc sulfate followed by one part of 0.5 *N* sodium hydroxide to three parts of sample. The resulting coagulum was separated by centrifugation, and the supernatant liquid was removed, acidified with hydrochloric acid, and treated with barium chloride to precipitate sulfate. The precipitate was removed by filtration, and the "Carbowax" Compound in the filtrate was then precipitated with additional barium chloride and silicotungstic acid.

The absorption of "Carbowax" Compounds 6000, 4000, 1540, and 1000 was studied. All these materials were used as 25% solutions. Fifty per cent and 12.5% solutions were tried in some cases, but the employment of the former was abandoned early as it caused a high incidence of diarrhea among the animals dosed. This variation in concentrations did not affect the results obtained. Usually 30 animals were dosed at a given time, and groups of 5 were killed at successive hourly intervals up to five hours after dosing. The tests included one control group that was sacrificed immediately.

Tabulation of the data accumulated from these experiments would be superfluous at this point. Statistical treatment of these results using a level of significance of $p = 0.01$ reveals that "Carbowax" Compounds 6000 and 4000 show no measurable absorption from the intestine of the rat over a five-hour period. The initial dose was recovered from the gastrointestinal tract with quantitative accuracy at each time interval up to this point. "Carbowax" Compound 1540 showed some slight absorption amounting to not more than a mean of 0.027 Gm./100 Gm. of body wt./hr., or less than 2% of the total dose in five hours. Similarly, "Carbowax" Compound 1000 showed an absorption amounting to not more than a mean of 0.022 Gm./100 Gm. of body wt./hr., or less than 2% of the total dose in five hours under the conditions of this experiment.

Injection Experiments with Humans.—Because of the obvious desirability of limiting manipulation on the subjects, experimentation on human subjects has been confined to "Carbowax" Compounds 1000 and 6000. Results obtained on these two extremes might well be expected to be representative of the intermediate members of the series. Six adult men have cooperated in the second phase of this work. These are H. F. S., age 43, wt. 86.4 Kg.; C. P. C., age 35, wt. 70 Kg.; C. B. S., age 29, wt. 82 Kg.; C. S. W., age 28, wt. 72.7 Kg.; C. A. W., age 29, wt. 68.2 Kg.; and R. T. S., age 30, wt. 66 Kg. These experiments usually began at 10 a. m. The subject had consumed a liquid breakfast at about 8 a. m., and small amounts of water were drunk at intervals during the succeeding two hours. The "Carbowax" Compound to be tested was prepared as a five per cent solution in physiological saline and sterilized in an autoclave. Exactly 20 ml. of this solution, representing 1 Gm. of the polyglycol, was then injected slowly into the antecubital vein. In order to check the accuracy of this measurement, an exactly similar quantity was delivered from the same syringe into a 500-ml. volumetric flask immediately prior to the injection. The flask was filled to the mark with distilled water, and an aliquot was withdrawn for determination of the quantity of polyglycol by actual analysis. The bladder was emptied just before the injection, and again every fifteen minutes for the first hour; later at extending intervals up to twelve hours when the experiment was terminated. No restrictions were placed upon lunch or dinner, and no attempt was made to regulate fluid intake. In many cases, urine excreted between the twelfth and twenty-fourth hour was collected and analyzed, but because less than 1% of the dose was found therein in early trials urine from this period was not collected in later experiments, and analyses are not tabulated.

The excretion curves of "Carbowax" Compounds 1000 and 6000 for two men are plotted in Fig. 1, and Tables I and II list the actual percentages of the total dose excreted in the urine during successive time intervals following the injection. These curves are quite representative of all the subjects. In the

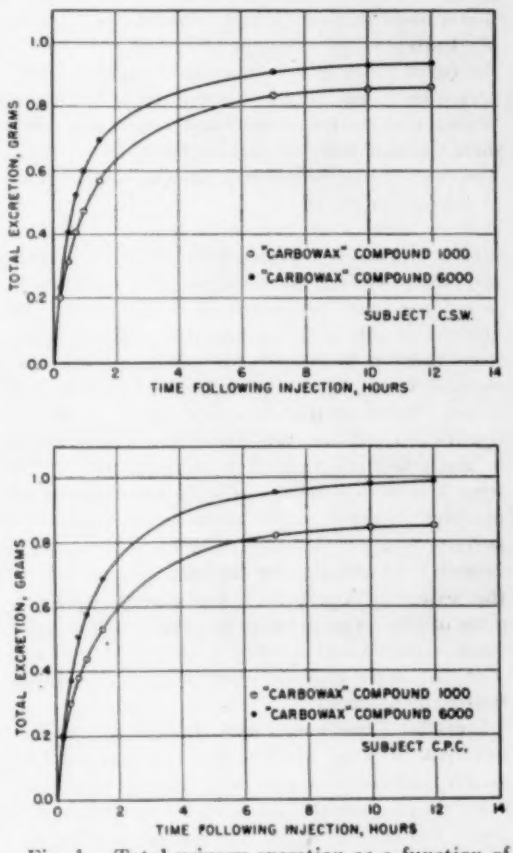

Fig. 1.—Total urinary excretion as a function of time following the intravenous injection of 1 Gm. of polyglycol.

TABLE I.—PERCENTAGE OF TOTAL DOSE EXCRETED IN THE URINE DURING SUCCESSIVE TIME INTERVALS FOLLOWING THE INTRAVENOUS INJECTION OF 1 GM. OF "CARBOWAX" COMPOUND 6000

Time Interval, Hr.	Excretion—Percentage of Total Injected						
	H. F. S., %	C. P. C., %	C. B. S., %	C. S. W., %	C. A. W., %	R. T. S., %	Mean, %
0 – 0.25	18.78	20.16	32.32	23.67	19.68	24.55	23.19
0.25– 0.5	18.06	17.40	21.90	16.81	18.02	22.03	19.04
0.5 – 0.75	12.16	13.06	12.59	12.29	16.30	12.19	13.10
0.75– 1.0	7.49	7.59	6.77	7.28	9.25	6.50	7.48
1.0 – 1.5	10.03	10.40	6.88	9.54	12.75	8.64	9.71
1.5 – 7.0	22.97	27.16	15.73	21.23	17.37	17.81	20.34
7.0 –10.0	3.10	2.06	1.86	2.09	1.86	1.75	2.12
10.0 –12.0	1.42	1.44	1.04	1.17	1.42	1.12	1.27
Total	94.01	99.27	99.09	94.08	96.65	94.59	96.25

TABLE II.—PERCENTAGE OF TOTAL DOSE EXCRETED IN THE URINE DURING SUCCESSIVE TIME INTERVALS FOLLOWING THE INTRAVENOUS INJECTION OF 1 GM. OF "CARBOWAX" COMPOUND 1000

Time Interval, Hr.	Excretion—Percentage of Total Injected						
	H. F. S., %	C. P. C., %	C. B. S., %	C. S. W., %	C. A. W., %	R. T. S., %	Mean, %
0 – 0.25	17.14	19.68	24.12	20.52	16.50	23.50	20.24
0.25– 0.5	12.68	10.01	12.22	11.05	11.30	10.05	11.22
0.5 – 0.75	9.28	8.56	8.84	9.34	11.48	8.87	9.40
0.75– 1.0	4.79	5.78	7.42	6.57	6.92	6.97	6.41
1.0 – 1.5	9.61	9.31	7.75	9.84	5.58	8.09	8.36
1.5 – 7.0	23.99	28.54	24.66	26.23	31.48	28.38	27.21
7.0 –10.0	2.09	2.76	1.28	2.13	1.75	0.70	1.78
10.0 –12.0	1.21	0.28	0.85	0.32	0.42	0.50	0.60
Total	80.79	84.92	87.14	86.00	85.43	87.06	85.22

case of "Carbowax" Compound 1000 the rate is slower and the total amount excreted less than that of "Carbowax" Compound 6000, but the shape of the curve is the same. Such differences are interpreted by us partially as a reflection of the greater tendency of the lower molecular weight polymer to diffuse more rapidly throughout the tissues, and to a lesser extent to a difference in the rate of destruction of the two by the tissues.

At no time in our experience has there been any evidence that the polyglycols are stored as such in the animal body, and therefore these amounts injected can only be destroyed or excreted. The amount considered as destroyed in any experiment may thereby be found by deducting the total amount found in the urine from the amount injected. Whether this difference, where small, represents actual destruction, other routes of excretion, or analytical uncertainty, is difficult to say. Be what it may, it is nevertheless an expression of our precision at recovering from the urine amounts of polyglycol that have found entry to the system, and as such it is adequate for the purpose at hand. In the case of "Carbowax" Compound 1000, the ratio of the mean amount destroyed to the mean amount excreted (147.8/852.2, or 0.1734) will subsequently enter into the calculation of the absorption of this material.

Ingestion Experiments with Humans.—Each subject took 10 Gm. of "Carbowax" Compound by mouth and the excretion was followed as before. For this purpose the polyglycol was dissolved in about 150 ml. of water. Urine was collected at hourly intervals for the first four hours following ingestion, and thereafter at longer periods up to twenty-four hours from the start of the experiment. In one instance (subject C. B. S.) urine was collected at the end of the first half-hour and then at fifteen-minute intervals up to one and three-quarter hours. The more frequent collections were for the purpose of a more detailed analysis of the actual absorption curve.

Quantities of "Carbowax" Compound 6000 in the urine of the six subjects following the ingestion of 10 Gm. were too small to be determined and it is concluded that this substance is to all practical purposes not absorbed from the gastrointestinal tract of the human subject. This conclusion is in agreement with what has been found to be true in the case of the rat.

The amounts (in percentage of the total dose) of "Carbowax" Compound 1000 excreted during successive time intervals following ingestion are recorded in Table III. Because of the relatively slight amounts involved, it was not considered worth while to present this information graphically as curves of excretion. Such a curve constructed for subject C. B. S. shows a slight initial delay in excretion which would undoubtedly be true for each of the others. This lag is not the result of a temporary retention of the solution in the stomach, but rather represents the time taken for the concentration in the tissues to rise.

The injection experiments have provided the factor 0.1734 representing the ratio between the amount of "Carbowax" Compound 1000 destroyed and the amount excreted. By its application, the amount destroyed in the ingestion experiments may be calculated. The amount destroyed added to the amount excreted equals the total amount absorbed.

Kinetics of the Absorption of "Carbowax" Compound 1000.—Thus far only the total amounts of "Carbowax" Compound excreted or absorbed have been considered. It is obvious that the actual curve of absorption should in no way resemble the curves of excretion shown in Fig. 1. The polyglycol continues to be excreted long after it has ceased to be absorbed since amounts temporarily stored in the body fluids are only slowly destroyed or excreted. The fact that excretion is not yet quite finished seven or eight hours after 1 Gm. has been injected is verification. A curve of absorption can be constructed from the following considerations. The amount absorbed up to any given moment is equal to the sum of (*a*) the amount excreted up to that moment, (*b*) the amount destroyed up to that moment, and (*c*) the amount remaining in the tissues at that moment.

The first figure is obtained by direct estimation. The second may be calculated by multiplying the first by the factor 0.1734 (in the case of "Carbowax" Compound 1000), assuming that this ratio is a constant whatever the time. The third, i.e., the amount in the tissues at any moment, is proportional to the rate of excretion at that moment and can be calculated from it according to the information portrayed in Fig. 2.

As a concrete example of this reasoning the amount of "Carbowax" Compund 1000 absorbed one hour after ingestion may be calculated as follows (subject C. B. S.):

(*a*) The amount excreted up to 1 hr. (by direct estimation) = 303 mg.

(*b*) The amount destroyed up to 1 hr. = 0.1734 × 303 = 52 mg.

(*c*) The amount remaining in the tissues at 1 hr.

The amount excreted between ¾ hr. and 1 hr. after ingestion = 83.24 mg. (by direct estimation); the amount similarly excreted between 1 and 1¼ hr. = 78.61 mg. Assuming the rate of excretion to be constant for this half hr., the rate of excretion in mg./min. at 1 hr. after ingestion = 5.4. From the smoothed curve of rate of excretion after injection (Fig. 2) this rate is found 48 min. from the start, at which time 440 × 0.1734 = 76 mg. have been destroyed.

Therefore 1000 − (440 + 76) = the amount in the tissues corresponding to a rate of excretion of 5.4 mg./min. = 484.

Amount in the tissues 1 hr. after ingestion = 484 mg.

Total amount absorbed = 839 mg.

As McCance (3) has pointed out, great accuracy cannot be expected from a calculation of this sort since certain assumptions are involved that are not strictly correct. It is assumed that the injected or

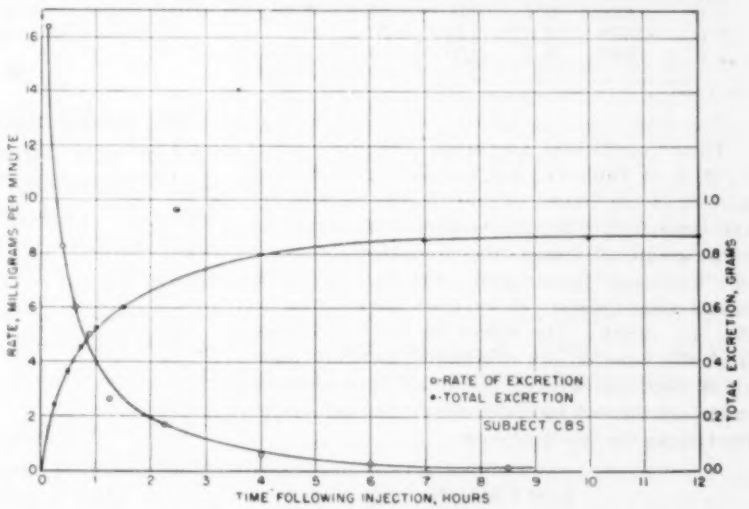

Fig. 2.—Rate and total urinary excretion as a function of time following the intravenous injection of 1 Gm. of "Carbowax" Compound 1000. In constructing the rate curve, each point is plotted in the center of the period during which that particular sample of urine was excreted.

TABLE III.—PERCENTAGE OF TOTAL DOSE EXCRETED IN THE URINE DURING SUCCESSIVE TIME INTERVALS FOLLOWING THE INGESTION OF 10 GM. OF "CARBOWAX" COMPOUND 1000

Time Interval, Hr.	Excretion—Percentage of Total H. F. S., %	C. P. C., %	C. B. S., %	C. S. W., %	C.A.W., %
0– 1	1.40	2.19	3.03	1.80	0.72
1– 2	1.14	2.56	1.86	3.16	3.34
2– 3	0.86	2.17	1.22	2.37	2.73
3– 4	0.39	0.89	0.47	1.00	1.37
4– 7	0.47	0.94	0.53	0.71	1.48
7–12	0.29	0.48	0.30	0.42	0.47
12–24	0.30	0.29	0.04	0.25	0.17
% of total excreted	4.85	9.52	7.45	9.71	10.28
% destroyed	0.84	1.65	1.29	1.68	1.78
% of total absorbed	5.69	11.17	8.74	11.39	12.06

absorbed material immediately distributes itself uniformly throughout the body. This supposition is not entirely true; however, it does appear from the curves of excretion that the intravenously injected polyglycols do leave the blood stream and diffuse into the organs with moderate rapidity. Secondly, the determination of rates in this manner is not quite accurate, particularly over parts of the curve where the rate is changing rapidly. This error may be minimized to a considerable extent by more frequent collection of samples during the first several hours of the experiment. Despite these assumptions, the general conclusions derived may be considered fundamentally reliable.

TABLE IV.—CALCULATION OF TOTAL QUANTITY ABSORBED AT SUCCESSIVE TIME INTERVALS FOLLOWING INGESTION OF 10 GM. OF "CARBOWAX" COMPOUND 1000 (Subject C. B. S.)

Time, Hr.	Amount Excreted, Mg.	Rate of Excretion, Mg./Min.	Amount Destroyed, Mg.	Amount in Tissues, Mg.	Amount Absorbed, Mg.
0.5	103	5.0	18	460	581
0.75	220	6.7	38	566	824
1.0	303	5.4	52	484	839
1.25	381	4.2	66	413	860
1.5	430	2.7	74	312	816
1.75	463	2.2	80	267	810
3.0	591	1.3	102	142	835
4.0	638	0.7	111	67	816
5.0	670	0.3	116	30	816
7.0	690	0.1	120	0	810

These calculations are summarized for subject C. B. S. in Table IV, and, considering the general pattern of the results (Table III) on all subjects, this example may be considered representative of the entire group. It appears therefrom that absorption of "Carbowax" Compound 1000 has practically ceased three-quarters of an hour after taking 10 Gm. by mouth. The values for total absorption from this time on are distributed normally, since all of them fall between 778 and 872, or within a range represented by their mean plus and minus three times the standard error.

DISCUSSION

It is of interest to note that, while the curves of excretion of both polyglycols with time are quite regular and comparable in all cases, they were frequently obtained at widely varying rates of urine flow both in the same experiment and in different experiments. In other words, the rate of excretion of water made little or no difference in the rate or amount of polyglycol excreted. This fact is consonant with the most recent theories of kidney function (4) which emphasize the remarkable constancy of glomerular activity in the normal man and dog as opposed to that in the frog and the intermediate position in this respect occupied by the rabbit. In the first two species variations in rate of urine flow do not involve any intermittency of glomerular function, but merely the resorption of variable quantities of water in the tubules. Fluctuations in the rate of water excretion, therefore, should not affect the concomitant rate of polyglycol excretion, except in so far as a scanty urine flow may afford greater opportunity for a passive back-diffusion of the polyglycol into the circulation.

It is apparent that the absorption of "Carbowax" Compound 1000 must commence as soon as the polyglycol is ingested, and it is striking that this absorption should proceed with moderate rapidity for about three-quarters of an hour and then almost or entirely cease while the greater part of the dose remains in the intestine. Two explanations of this finding present themselves. The simpler is that, since the polyglycol is actually a mixture, this degree of absorption may represent the uptake of a low molecular weight component or components, to which the intestine is freely permeable. This line of reasoning gains some support from the fact that the higher molecular weights as represented in "Carbowax" Compounds 4000 and 6000 are not absorbed. A second, and to us a more plausible explanation, would be that the site of absorption is very probably a limited region high in the small intestine, and that the ingested polyglycol passes this region before any considerable amount of absorption has occurred.

SUMMARY

The gastrointestinal absorption of a series of four solid polyethylene glycols ("Carbowax" Compounds) has been studied by Cori's method. "Carbowax" Compounds 4000 and 6000 showed no absorption from the intestine of the rat over a five-hour period, while "Carbowax" Compounds 1000 and 1540 showed a slight absorption amounting to less than 2 per cent of the total dose during the same period.

Both "Carbowax" Compound 6000 and "Carbowax" Compound 1000 are excreted readily when injected intravenously in man. In a series of six adult male subjects receiving 1 Gm. of "Carbowax" Compound 6000 each by this route, an average of 96 per cent of the polyglycol was recovered from the urine within the following twelve hours. Under similar conditions, recoveries of "Carbowax" Compound 1000 averaged 85 per cent.

No urinary excretion of "Carbowax" Compound 6000 could be detected in any of five subjects receiving 10 Gm. of this material by mouth. Following the ingestion of

an equal amount of "Carbowax" Compound 1000, an average of 8 per cent of the dose was excreted in the urine within the following twenty-four hours.

The calculation of the actual intestinal absorption of these compounds in the human from a consideration of their urinary excretion following intravenous injection and oral administration is outlined, and certain features of this process are discussed.

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The Toxicity of High Molecular Weight Polyethylene Glycols; Chronic Oral and Parenteral Administration*

By HENRY F. SMYTH, JR., CHARLES P. CARPENTER, and C. BOYD SHAFFER

Results of administering oral doses of high molecular weight polyethylene glycols to rats over a two-year period are reported. The single dose toxicity for several of these compounds by intraperitoneal administration to rats and intravenously to rabbits was also determined.

POLYETHYLENE GLYCOLS of high average molecular weight, sold under the tradename of "Carbowax" compounds, have been widely utilized in preparations intended for external application. The "Carbowax" compounds and the lower polyethylene glycols consist of mixtures of similar molecules within a band of molecular weights, the average of which is used in characterizing the compound.

We have previously reported (1) that "Carbowax" compounds 1500 and 4000 manifest little irritation upon human skin, possess a very low order of acute toxicity to rats and rabbits, and do not penetrate the intact rabbit skin. Over a ninety-day period albino rats were not killed by daily dosages of 23 and 19 Gm./Kg., respectively, of the two materials administered in the drinking water, but the least daily dosages revealing no injurious effect whatever were 2.0 and 0.04 Gm./Kg., respectively. In this subacute study injury from low dosages consisted of retarded growth and light cloudy swelling of renal tubular elements. Rabbits received 30 daily doses by stomach tube of up to 20 Gm./Kg. each of "Carbowax" compound 4000 without fatalities or development of microscopic pathology in the liver or kidney, although glycogen in the former organ was seen to be reduced. The daily doses of 10 Gm./Kg. did not interfere with the function of either of these organs.

In connection with similar compounds of lower molecular weight, polyethylene glycols 200, 300, and 400, we have reported (2) that the acute and sub-acute oral toxicity to rats and rabbits is of a low order, irritation to the skin of humans and rabbits is comparable to that of other materials commonly applied to the skin, and skin penetration during inunction upon rabbits produced little evidence of sub-acute toxicity.

More recently Shaffer and Critchfield (3) have devised a method for the quantitative estimation of polyglycols in body fluids, and have subsequently shown (4) that absorption from the digestive tract of the rat and man is negligible. Single doses of "Carbowax" compound 6000 injected intravenously in humans were rapidly and quantitatively excreted by the kidneys. In the case of "Carbowax" compound 1000, all but approximately 15 per cent of such doses could be recovered from the urine within the following twenty-four hours.

* Received Dec. 26, 1946, from the Mellon Institute of Industrial Research, Pittsburgh, Pa.

The present report supplements the available information upon "Carbowax" compounds with the results of oral doses administered to rats over a two-year period, together with certain observations upon parenteral administration.

METHOD

An equal number of male and female Wistar strain albino rats born within a two-week period were obtained from a commercial breeder. During three to four weeks of observation the most vigorous animals were selected. Seventy-two of each sex were distributed at random among nine groups with 8 males and 8 females in each. Four groups received aqueous solutions of "Carbowax" compound 1500 in place of drinking water, in concentrations of 2, 0.4, 0.08, and 0.02%, four received "Carbowax" compound 4000 in concentrations of 0.08, 0.02, 0.005, and 0.00125%, while the ninth group was given plain water. These fluids were freely available to the animals in non-fouling siphon water bottles subsequent to the time the rats reached forty-seven to fifty-nine days of age. The diet consisted of Purina Dog Chow Checkers supplemented with oranges, kale, carrots, liver, and marrow bones to lend variety and reduce cannibalism.

The rats were kept 4 to 6 in a cage and a running record of fluid consumption was maintained to allow calculation of the dosages. The weighted mean dosage levels over the two-year period of "Carbowax" 1500 were 1.69, 0.27, 0.059, and 0.015 Gm./Kg./day and of "Carbowax" 4000 they were 0.062, 0.017, 0.0036, and 0.00085 Gm./Kg./day.

The animals were allowed to breed and a record was kept of litters born to each female. Some pups from early litters were used to establish an F_1 generation of three males and three females at each dosage level, and later an F_2 generation of the same size. A total of 28 animals was studied at each dosage level. The results obtained were considered together after demonstrating statistically that there was no significant difference in the responses of the three generations.

The animals were weighed weekly during the period of active growth and monthly thereafter. The dosages were maintained unchanged until the parental generation was seven hundred seventy-five to seven hundred eight-eight days old, at which time the survivors were sacrificed for examination. Blood counts were performed at intervals of about three months upon some rats in each group. Pooled urine samples were examined for sugar and albumen after three, six, and twenty-four months.

RESULTS

The study lasted for the major part of the normal life span of the rats, and in the course of this time 55% of the animals died. The criteria considered in the determination of toxic response to the dose were mortality, frequency of extraneous infection, life span, fluid consumption, rate of increase in body weight and length, fatness index (grams weight per millimeter body length), kidney and liver weights, frequency and size of litters, blood cytology, urinary albumen and reducing sugars, occurrence of neoplasms, and micropathology. The organs examined were adrenal, heart muscle, small intestine, kidney, liver, pancreas, spleen, testis, and occasionally stomach wall and leg muscle. All numerical data were evaluated statistically by the "t" test (5) with the fiducial point at the significant level of 1 in 20 for "p." The only criterion which revealed any relation between dosage and response was the rate of increase in body weight. The highest and next highest dosages of "Carbowax" compound 1500 and the highest of "Carbowax" compound 4000 reduced growth so that the body weight curves were consistently below those of the groups receiving lower dosages and the control group. At one year of age the difference between the mean weights of the affected and unaffected groups was approximately 9%.

Because a large proportion of the animals were senile at death or sacrifice, the finding of many neoplasms was not surprising. These were not distributed in proportion to the dosage and hence are not considered a manifestation of injurious action of "Carbowax" compounds. Ratcliffe (6) has reported that, in the Wistar Institute rat colony, chronic pneumonia is found in 75% of rats one year of age or older, and he warned that regenerative lung processes may yield a picture that can be mistaken for malignancy. He found only 3% of one-year old rats of the Wistar strain to reveal true neoplasms. In contrast to this statement, Fitzhugh (7) has said that, among the Food and Drug Administration's Osborne and Mendel strain of rats, lymphosarcoma of the lung was very frequently seen. In the present study 40% of the rats over one year old had neoplastic-like processes; the majority were in the lung, similar to the lymphosarcoma seen by Fitzhugh, often accompanied by metastasis at a distance. Because the incidence of these abnormalities could not be correlated with dosage it is of no concern to the immediate problem whether they were all malignancies, or whether some were merely the regenerative processes described by Ratcliffe.

Aside from neoplasms, the only notable condition found upon autopsy consisted of soft aggregates of nearly pure protein in 10 bladders. These were distributed among the groups, dosed and control, independently of dosage, and were usually related to senile degenerative changes in the kidney tubules which were associated with the lung condition diagnosed as lymphosarcoma.

There were no other micropathological conditions which could be attributed to the feeding of "Carbowax" compounds. At the low dosages administered over this two-year period the kidney and liver changes reported from greater amounts given for ninety days (1) were not seen.

PARENTERAL ADMINISTRATION

The mild effects from single and long continued repeated oral doses coupled with evidence of very slight absorption from the digestive tract (4) pointed to the need for more information upon the toxicity of parenterally injected "Carbowax" compounds.

Listed below are the LD_{50} values for the solid "Carbowax" compounds when aseptically administered as 50% aqueous solutions by intraperitoneal injection in Wistar strain male albino rats weighing 90 to 120 Gm. The solutions had been sterilized by 15 pounds steam pressure for twenty minutes.

"Carbowox" compound 1000 . . . 15.57 (12.84 to 18.89)Gm./Kg.
"Carbowax" compound 1500 . . . . 17.72 (15.34 to 20.48) Gm./Kg.
"Carbowax" compound 1540 . . . . 15.39 (13.66 to 17.38) Gm./Kg.
"Carbowax" compound 4000 . . . . 11.55 (9.59 to 13.90) Gm./Kg.
"Carbowax" compound 6000 . . . . 6.79 (3.56 to 12.99) Gm./Kg.

Eighty per cent of the rats that succumbed died within thirty hours of the dose; survivors were observed for fourteen days. Microscopically most livers were normal, with marked cloudy swelling being the most severe injury in any of the 258 rats examined. No trace of "Carbowax" compound 4000 could be found analytically in the peritoneal cavity fourteen days after the doses. Other studies (4) showed that intravenously injected polyglycol must leave the body through the kidney and presumably this intraperitoneally administered material followed a similar route. Yet the majority of the kidneys were normal, although a small percentage revealed cloudy swelling of the tubular epithelium, sometimes accompanied by nuclear degeneration.

As a preliminary to the work of Shaffer and Critchfield upon intravenous doses of "Carbowax" compounds to human subjects (4) similar doses were administered to rabbits. It was early found that red blood cells of rabbit, dog, and man were crenated and clumped by each of the compounds listed above in concentrations of 10% or greater. In the case of "Carbowax" compound 4000 this finding was confirmed *in vivo* upon rabbits which received intravenous infusions of 10% solutions. In two animals that died of lung hemorrhage, the blood when examined microscopically was seen to contain abundant clumps of cellular elements. Five per cent solutions did not manifest this objectionable action.

Rabbits survived the slow intravenous injection of 10 Gm./Kg. of the five solid "Carbowax" compounds listed above. Injection was performed by machine at a uniform rate of 2.5 ml. of a 5% solution per minute. The animals were sacrificed after fourteen days and all livers and kidneys were found to be normal microscopically with the exception of the kidney of one rabbit which had received "Carbowax" compound 6000. This organ revealed light cloudy swelling of both loop and convoluted tubules.

Daily intravenous injections of 1 Gm. of the 5 solid compounds and of polyethylene glycols 200, 300, and 400, as 5% solutions in 0.85% sodium chloride autoclaved for twenty minutes at 15 pounds pressure, were made in groups of 3 to 9 rabbits each, six days a week for a total of 30 doses. The only deaths that resulted from the doses were as follows:

"Carbowax" compound 6000, 1 of 5 died;
"Carbowax" compound 4000, 1 of 9 died;
Polyethylene glycol 400, 1 of 5 died.

All livers and kidneys were examined in microscopic section. All were normal except for cloudy swelling of various degrees in both organs in 4 of 9 animals from "Carbowax" compound 4000, 1 of 3 from 1000, 1 of 5 from polyethylene glycol 400, and 2 of 4 from 200.

After the injections were well underway it was found that steam sterilization of the aqueous solutions had produced small but detectable amounts of formaldehyde, possibly from hydrolysis of formal compounds present as impurities. There is a possibility that this foreign substance, which did not exceed 0.02% in the solutions, accounted for some of the evidences of injury noticed.

DISCUSSION

Fitzhugh and Nelson (8) have reported upon the chronic oral toxicity of diethylene and triethylene glycol to rats and they have suggested that chronic toxicity may decrease as one ascends to higher molecular weights in the ethylene series of polyglycols. The present study casts doubt on this inference.

Single oral doses to rats and guinea pigs (1) have indicated "Carbowax" compound 4000 to be less acutely toxic than 1500. The respective figures for rabbits are approximately 76 Gm./Kg. and 29.8 (25.5 to 34.7) Gm./Kg., showing the same relation as for the two other species. The two-year oral doses here presented support the ninety-day series (1) in demonstrating "Carbowax" 4000 to possess the greater cumulative action.

Formation of formaldehyde while the "Carbowax" solutions were in the drinking water bottles of the rats cannot be used to explain the greater toxicity of 4000 because a few trials revealed that "Carbowax" compound 1500 forms more aldehyde than does 4000, while the concentration produced by neither exceeds a few thousandths of 1 per cent.

Shaffer and Critchfield (4) were unable to

estimate intestinal absorption of "Carbowax" 1500 because it is a blend of 1540 and polyethylene glycol 300, the latter of which is imperfectly precipitated by their analytical reagent. Their data show that 1540 is absorbed to a greater extent than 4000, however, and it is reasonable to assume that the same holds true for 1500. We are therefore faced with an anomalous situation in which the more readily absorbed of these very similar compounds has the lesser chronic oral toxicity.

Further work is in progress in an attempt to explain the chronic toxicity of "Carbowax" 4000, which as a high molecular weight compound not appreciably absorbed from the digestive tract would be expected to possess a low degree of cumulative action.

SUMMARY

Dosages of 0.06 Gm./Kg./day of "Carbowax" 1500 or of 0.02 Gm./Kg./day of "Carbowax" compound 4000 did not injure albino rats when administered in the drinking water over a two-year period. Larger dosages retarded growth.

The single dose intraperitoneal toxicity of 8 polyethylene glycols to rats is low, and rabbits tolerated large single intravenous injections of the 5 solid compounds. Repeated intravenous doses injured only a small proportion of those receiving them.

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Book Reviews

Limit Tests for Impurities, by F. REIMERS and K. R. GOTTLIEB. (Text in Danish with an English summary, 27 pp.) Vol. I of Contribution from the Danish Pharmacopœia Commission. Einar Munksgaard, Copenhagen, Denmark, 1946. 198 pp. 15 x 25 cm. Price, d. kr. 10.

"In modern pharmacopœias we often use controls in connection with purity tests, starting from the following assumption. If the quantity and concentration of the solution to be examined, the quantity of reagent, and the maximum strength of the reaction obtained are given, also the maximum quantity of impurity permissible must be determined simultaneously."

The Danish Pharmacopœia Commission, Chemistry Subcommittee set out to establish this assumption and the results are found in this book. Such common tests as limit for H ion, chloride, sulfate, and nitrate were studied in considerable detail as to order of adding reagents, temperature, time, *p*H, and the presence of other salts. Of interest are such findings as the necessity of using a "sowing solution" of a known, minute quantity of barium sulfate in the barium chloride solution to assure reproducible results in the limit test in sulfate.

The revision committees of the U. S. P. and the N. F. would do well to re-investigate some of their tests in light of these suggestions.—MELVIN W. GREEN.

Ether for Anesthesia, by F. REIMERS. (In Danish with a summary in English, 12 pp).. Einar Munksgaard, Copenhagen, Denmark, 1943. 311 pp. 15 x 25 cm.

The author has made a thorough study of the impurities in ethyl ether, their origin and methods of detecting them and methods of preserving the ether. Experiments were made on such factors as light, type of container, etc., as they affect stability. The author concludes that the fuchsin-sulfurous acid test (containing 0.1 per cent pyrogallol) is the most sensitive for aldehyde and the test with neutral potassium iodide and starch is best for peroxide.—MELVIN W. GREEN.